

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
4 August 2005 (04.08.2005)

PCT

(10) International Publication Number
WO 2005/071069 A1

(51) International Patent Classification: **C12N 7/04**,
A61K 39/12, C12N 15/62, C07K 14/08, 19/00, C12N
15/87, 15/86, 5/10

Daniel [ES/ES]; Serrano, 117, E-28006 Madrid (ES).
RODRIGUEZ FERNANDEZ-ALBA, Juan Ramón
[ES/ES]; Ronda de Poniente, 4-2° C-D, E-28006 Tres
Cantos - Madrid (ES).

(21) International Application Number:
PCT/EP2005/000695

(74) Agent: **ARIAS SANZ, Juan**; ABG Patentes, S.L., Orense,
68, 7th floor, E-28020 Madrid (ES).

(22) International Filing Date: 21 January 2005 (21.01.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
P200400120 21 January 2004 (21.01.2004) ES

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(71) Applicants (for all designated States except US):
CONSEJO SUPERIOR DE INVESTIGACIONES
CIENTÍFICAS [ES/ES]; Serrano, 117, E-28006 Madrid
(ES). **BIONOSTRA, S.L.** [ES/ES]; Ronda de Poniente,
4-2° C-D, E-28760 Tres Cantos - Madrid (ES).

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,
SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **RODRIGUEZ**
AGUIRRE, José Francisco [ES/ES]; Serrano, 117,
E-28006 Madrid (ES). **RUIZ CASTON, José** [ES/ES];
Serrano, 117, E-28006 Madrid (ES). **GONZALEZ**
DE LLANO, María Dolores [ES/ES]; Serrano, 117,
E-28006 Madrid (ES). **RODRIGUEZ AGUIRRE, María**
Dolores [ES/ES]; Serrano, 117, E-28006 Madrid (ES).
BLANCO CHAPINAL, Soledad [ES/ES]; Serrano, 117,
E-28006 Madrid (ES). **OÑA BLANCO, Ana María**
[ES/ES]; Serrano, 117, E-28006 Madrid (ES). **SAUGAR**
GOMEZ, Irene [ES/ES]; Serrano, 117, E-28006 Madrid
(ES). **ABAITUA ELUSTONDO, Fernando** [ES/ES];
Serrano, 117, E-28006 Madrid (ES). **LUQUE BUZO,**

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: CHIMERIC EMPTY CAPSIDS OF THE INFECTIOUS BURSAL DISEASE VIRUS (IBDV), OBTAINMENT
PROCESS AND APPLICATIONS

(57) Abstract: The chimeric empty capsids of the infectious bursal disease virus (IBDV), are constituted by assembly of (i) IBDV
pVP2 proteins and (ii) fusion proteins comprising a region A constituted by the IBDV VP3 protein bound to a region B constituted
by a heterologous polypeptide comprising a polypeptide of interest, such as a polypeptide useful in vaccination, therapy or diagnosis.

WO 2005/071069 A1

CHIMERIC EMPTY CAPSIDS OF THE INFECTIOUS BURSAL DISEASE VIRUS (IBDV), OBTAINMENT PROCESS AND APPLICATIONS

FIELD OF THE INVENTION

5 The invention is related to the production of chimeric empty particles of the infectious bursal disease virus (IBDV) and their applications.

BACKGROUND OF THE INVENTION

10 Viral particles are structures specialized in the packaging and incorporating in vehicles of nucleic acids and proteins. A general feature of viral particles is their excellent ability for the immune response stimulation of the host. These properties make viral particles agents of extraordinary interest for the development both of intracellular delivery systems and for the generation of particulate vaccines. The use of different genetic expression systems has facilitated the production of viral-like particles or empty viral capsids (VLPs) of different types
15 of viruses (US patent 6,458,362 Casal, et al. 2002. Recombinant VP2 parvoviral pseudo-particles encoding CTL or T-helper cell epitopes; US 5,932,426 Baralle, et al. 1999. Molecular presenting system; US 6,602,705 Barnett, et al. 2003 Expression of HIV polypeptides and production of virus-like particles). The genetic manipulation of these expression systems in turn allows the production of VLPs containing heterologous amino acid sequences coming
20 from proteins other than those forming the native viral capsid. These VLPs are generically called heterotypical, recombinant or chimeric VLPs (CVLPs). CVLPs have mainly been used for two purposes: (i) generation of multivalent vaccines by means of immunologically relevant heterologous peptides (Kingsman, A. J., N. R. Burns, G. T. Layton, and S. E. Adams. 1995. Yeast retrotransposon particles as antigen delivery systems. *Ann. N. Y. Acad. Sci.* 754: 202–
25 213; Lo-Man, R., P. Rueda, C. Sedlik, E. Deriaud, I. Casal, and C. Leclerc. 1998. A recombinant virus-like particle system derived from parvovirus as an efficient antigen carrier to elicit a polarized Th1 immune response without adjuvant. *Eur. J. Immunol.* 28: 1401–1407; Qiu, Z., D. Ou, H. Wu, T. C. Hobma, and S. Gillam. 1994. Expression and characterization of virus-like particles containing rubella virus structural proteins. *J. Virol.* 68: 4086–4091); and
30 (ii) modification of the tropism by means of insertion of amino acid sequences involved in interactions with receptor-ligand (Schmidt, U., Rudolf, R., and Böhm, G. 2001. Binding of external ligands onto an engineered virus capsid. *Prot. Eng.* 14: 769–774; Shin, Y.C., and Folk,

W.R. 2003. Formation of polyoma virus-like particles with different VP1 molecules that bind the urokinase plasminogen activator receptor. *J. Virol.* 77: 11491-11498).

CVLPs are generally obtained by means of the expression of the viral protein(s) responsible for the formation of the viral capsid, fused to the region encoding the polypeptide of interest.

The infectious bursal disease virus (IBDV), belonging to the *Birnaviridae* family, infects different bird species and is directly responsible for a severe immunosuppressive disease causing important economic losses in the world poultry industry.

IBDV particles are icosahedral, with T=13 symmetry, they lack an envelope and are formed by a single protein layer. Up until now, the approaches aimed at obtaining an atomic model for IBDV particles have failed. As a result, the structural information available is based on three-dimensional models generated from images obtained by electron cryomicroscopy of the purified virus and of the VLPs. Based on these studies, it has been verified that the outer surface of the particle is formed by a continuous lattice of 260 trimers of the VP2 protein (37 kDa) organized in five different formations. The inner face of the particles contains 200 trimers of the VP3 protein (29 kDa), the latter, independent from one another, are bound to the basal area of the VP2 trimers. It has been suggested that a third polypeptide, VP4 (28 kDa), could also be part of the particles, being located at the base of the pentamers forming the vertices of the icosahedral structure.

The VP2, VP3 and VP4 polypeptides are produced from the proteolytic processing of a polypeptide precursor of a size of 109 kDa. This precursor is auto-catalytically processed, releasing the pVP2 (48 kDa), VP3 and VP4 polypeptides. The VP4 domain, which is located in the central region of the polyprotein, belongs to the Lon protease family and is responsible for the proteolytic cleavage. The pVP2 and VP3 polypeptides are directly responsible for the capsid assembly. The pVP2 product undergoes a last cleavage at its C-terminal end before giving rise to the mature form of the protein, VP2, which is the one found in purified particles (Da Costa, B., Chevalier, C., Henry, C., Huet, J. C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of Virology* 76:2393-2402). This pVP2 processing is necessary for the correct formation of the capsids and requires the presence of VP3, although the responsible protease has not yet been identified (Maraver, A., Oña, A., Abaitua, F., González, D., Clemente, R., Díaz-Ruiz, A., Caston, J. R., Pazos, F. & Rodríguez, J. F. (2003). The oligomerization domain of VP3, the scaffolding protein of

infectious bursal disease virus, plays a critical role for capsid formation. *Journal of Virology* 77:6438-49).

In general terms, morphogenesis is a vital process for the viral cycle requiring successive steps associated to modifications in the polypeptide precursors. As a result, viruses have developed strategies allowing the sequential and correct interaction between each one of their components. One of these strategies, frequently used by icosahedral viruses, is the use of polypeptides coming from a single polyprotein as the base of their structural components. In these cases, the suitable proteolytic processing of said polyprotein plays a crucial role in the assembly process.

This concept for the assembly of IBDV capsids has been demonstrated in earlier work (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054). Expression of the gene encoding for the IBDV polyprotein in eukaryotic cells gives rise to the formation of VLPs that are completely morphologically and biochemically indistinguishable from the IBDV virions. It has also been shown that the assembly of the capsids requires only the synthesis and correct processing of the viral polyprotein and is independent of the presence of the viral genome or of other proteins encoded by the viral genome, such as VP5 and VP1.

The results obtained to date from the IBDV gene expression in different recombinant systems has allowed concluding that: i) the assembly process is independent of the presence of genetic material of the virus, ii) only the polypeptides encoded by the polyprotein gene are necessary for the assembly, and iii) the assembly requires a coordinated interaction between the pVP2 and VP3 polypeptides.

However, it must be indicated that it is not known if the VP2/VP3 interaction is established between VP2 and VP3 domains of the polyprotein precursor when it has not yet undergone modifications, or on the contrary, if this interaction occurs after the processing of the precursor. Furthermore, current information does not exclude the possibility that VP4 could play a relevant role in the morphogenesis of the viral capsid. In fact, IBDV VLPs formed by assembly of the IBDV VP2, VP3 and VP4 proteins have been disclosed (US 6,528,063, US 5,788,970 and JP 5194597).

The work developed by the same inventors has enabled establishing systems for obtaining IBDV VLPs using different eukaryotic expression vectors. These vectors have been used for IBDV polyprotein expression in the absence or presence of the viral VP1 RNA

polymerase. The biochemical characterization of purified VLPs demonstrates that they contain pVP2, VP2 and VP3 proteins when only the viral polyprotein is expressed, and the pVP2, VP2, VP3 and VP1 proteins when the simultaneous expression of the polyprotein and viral RNA polymerase is carried out (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79: 1047-1054; Martínez-Torrecuadrada, J. L., Castón, J. R., Castro, M., Carrascosa, J. L., Rodríguez, J. F. & Casal, J. I. (2000). Different architectures in the assembly of infectious bursal disease virus capsid proteins expressed in insect cells. *Virology* 278: 322-331; Maraver, A., *et al.*, (2003) cited *supra*; Lombardo, E., Maraver, A., Castón, J. R., Rivera, J., Fernández-Arias, A., Serrano, A., Carrascosa, J. L. & Rodríguez, J. F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73: 6973-6983).

On the other hand, patent application WO 02/088339 discloses IBDV viral-like particles formed by assembly of chimeric proteins comprising the IBDV polyprotein bound at its carboxyl terminal end to a polypeptide.

However, CVLPs solely based on IBDV pVP2 and VP3, the latter VP3 protein being fused to a polypeptide of interest, or their potential as vehicles of products of interest, have not been previously disclosed.

SUMMARY OF THE INVENTION

The invention is faced with the problem of providing new tools for incorporating in vectors or vehicles products of interest, such as molecules with biological activity, for example drugs, polypeptides, proteins, nucleic acids, etc.

The solution provided by this invention is based on it being possible to generate, based on the simultaneous expression of the IBDV pVP2 and VP3 proteins, the latter genetically modified to include a nucleotide sequence encoding for a heterologous polypeptide comprising a polypeptide of interest, IBDV chimeric empty capsids (CVLPs). The resulting CVLPs are formed by assembly of (i) IBDV pVP2 proteins and (ii) fusion proteins comprising a region A constituted by the IBDV VP3 protein bound to a region B constituted by a heterologous polypeptide comprising a polypeptide of interest, wherein said region B is bound to the amino- or carboxy- terminal end of said IBDV VP3 protein. These CVLPs can be used for therapeutic,

preventive or diagnostic purposes, etc., for example in the manufacture of gene therapy vectors or vaccines.

The inventors had previously found that when IBDV VPX (pVP2) and VP3 proteins are expressed from independent genes, empty IBDV particles (VLPs) are formed. These VLPs are structurally identical to those obtained by means of expression of the ORF corresponding to the IBDV polyprotein. As part of the development of new vaccination strategies, the possibility of using this IBDV VLP production strategy for obtaining CVLPs which contained heterologous amino acid sequences, corresponding to peptides of interest, such as a histidine tag (Example 1), GFP (Example 2) and finally peptides involved in immune response induction (Example 3), was analyzed. As is demonstrated, the fusion of heterologous sequences in these constructs is not an obstacle for the formation of CVLPs.

As a study model of peptide-transporting CVLPs involved in an immune response, the possibility of obtaining CVLPs which had the sequence corresponding to the CD8 epitope (E-CD8) of the malaria CS protein (*Plasmodium yoelii*) was approached. This epitope is responsible for the CD8-specific cellular immune response induction against this pathogen, which can be quantified by means of the ELISPOT technique in splenocyte cultures from BALB/c mice (Example 3).

In summary, the obtained results clearly show that: (i) the expression system used allows obtaining IBDV CVLPs containing heterologous amino acid sequences; and (ii) immunization with said IBDV CVLPs induces a specific immune response to the heterologous amino acid sequence present in the CVLPs.

Therefore, an aspect of the present invention is related to an IBDV chimeric empty capsid characterized in that it is constituted by assembly of (i) IBDV pVP2 proteins and (ii) fusion proteins comprising a region A constituted by the IBDV VP3 protein bound to a region B constituted by a heterologous polypeptide comprising a polypeptide of interest.

A further aspect of this invention is related to a process for producing said IBDV CVLPs provided by this invention, based on the gene coexpression of said IBDV pVP2 and fusion proteins as two independent genes.

The nucleic acids, gene constructs, expression systems and host cells developed for implementing said process of producing said IBDV CVLPs, as well as their use for the production of said IBDV CVLPs, constitute further aspects of the present invention.

Said IBDV CVLPs have the ability to incorporate in vectors or vehicles products of interest such as molecules with biological activity, for example, polypeptides, proteins,

nucleic acids, etc. In a particular embodiment, said IBDV CVLPs can internally incorporate in vehicles antigens and immune response inducers in animals or humans to whom it is supplied, whereby they can be used in the manufacture of vaccines against human and animal diseases caused by viruses, bacteria, parasites or any other type of microorganism or against tumor diseases. In another particular embodiment, said IBDV CVLPs are used in the manufacture of gene therapy vectors.

Therefore, in a further aspect, the present invention is related to the use of said IBDV CVLPs in the manufacture of medicaments, such as vaccines and gene therapy vectors. Said vaccines and vectors constitute further aspects of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. (a) The diagram schematizes the proteolytic processing steps necessary for the formation of mature VP2 and VP3 capsid proteins from the polyprotein precursor. (b) The diagram reflects different genetic constructs derived from the IBDV polyprotein described up until now, as well as the structures produced by means of its expression in different heterologous systems. The numbers indicate the position corresponding to the first and last amino acid residue of the polyprotein present in each one of the constructs. The lower portion of the figure shows images obtained by means of transmission electron microscopy of the structures obtained by means of expression of the different constructs. The bar corresponds to 50 nm. The data has been taken from the following literature references: Fernández-Arias *et al.*, (1998), cited *supra*; Maraver *et al.*, (2003), cited *supra*; Martínez-Torrecuadrada *et al.*, (2000), cited *supra*; Castón *et al.*, 2001. C terminus of infectious bursal disease virus major capsid protein VP2 is involved in definition of the t number for capsid assembly. *Journal of Virology* 75, 10815-10828.

Figure 2. Microscopic analysis of H5 insect cells coexpressing pVP2 and VP3. The pVP2 and VP3 protein subcellular distribution was analyzed by means of confocal immunomicroscopy. Cells infected with the FB/pVP2 (a), FB/VP3 (b), or FBD/pVP2-VP3 (c-e) rBVs were incubated with rabbit anti-pVP2 serum and rat anti-VP3 serum. Then the cells were incubated with goat anti-rabbit IgG serum coupled to Alexa 488 (red) and goat anti-rat IgG serum coupled to Alexa 594 (green). The cores were stained with To-Pro 3 (blue). (e) Overlaying of the images shown in panels (c) and (d). Electron microscopy images corresponding to sections of H5 cells infected with different genetic constructs derived from the IBDV polyprotein. (f) Low-magnification image of an H5 cell infected with

a parental Fb virus. The insert corresponds to an enlarged detail of the area indicated by the box. (g) Low-magnification image of an H5 cell infected with the FBD/pVPX-VP3 virus. The insert corresponds to an enlarged detail of the area indicated by the box. (h) High-magnification image of an H5 cell infected with the FBD/pVPX-VP3 virus showing the formation of IBDV structures in detail. (i) High-magnification image of a BSC1 cell infected with the VTLacOI/POLY recombinant vaccine virus showing structures similar to those detected in panel (h). The bars indicate 600 nm (panels f and g) and 200 nm (panels h and i).

Figure 3. Structural and biochemical characterization of the structures derived from IBDV produced in insect cells coinfecting with the FB/pVP2 + FB/his-VP3 recombinant baculoviruses (rBV). Cells coinfecting with FB/pVP2 and FB/his-VP3 rBVs, or infected with the FBD/Poly-VP1 or FB/pVP2 virus, were used to purify structures derived from IBDV by means of centrifugation on sucrose gradients. Panels (a), (b), and (c) show transmission electron microscopy images corresponding to fraction 4 of the gradients obtained from infections with FBD/Poly-VP1, FB/pVP2+FB/his-VP3, and FB/pVP2, respectively. Panel (d) shows the results of a Western blot analysis of the sucrose gradients corresponding to the cultures infected with FBD/Poly-VP1 and FB/pVP2+FB/his-VP3, respectively. The total extracts (input) and the different fractions of the sucrose gradients (fraction F1 corresponds to the bottom of the gradient) were analyzed by means of Western blot using specific sera against the IBDV VP1, pVP2, VP3, and VP4 proteins, respectively. The molecular mass of the immunoreactive polypeptides is indicated in kDa.

Figure 4. Biochemical and structural characterization of IBDV VLPs produced in *S. cerevisiae* transformed with the plasmid pESCURA/pVP2-VP3-GFP. A *S. cerevisiae* culture transformed with the plasmid pESCURA/pVP2-VP3-GFP was grown at 30°C in a medium supplemented with the inducer galactose. At 18 hours, the culture was harvested and centrifuged. The resulting sediment was processed by means of fractioning in a 25-50% linear sucrose gradient. A) Biochemical analysis of samples corresponding to the sediment before fractioning (T) as well as the different fractions of the sucrose gradient. The samples were analyzed by means of SDS-PAGE and Western blot using specific antibodies against VP3 (anti-VP3) and pVP2 (anti-pVP2) proteins. The arrows indicate the positions of the immunoreactive bands corresponding to the VP3-GFP (61 kDa) and pVP2 (48 kDa) proteins, respectively. B) The structural analysis of the obtained samples was carried out by means of TEM. The image corresponds to a micrograph obtained from an aliquot corresponding to the mixture of fractions 7, 8 and 9 of the sucrose gradient. The sample was

stained with uranyl acetate and observed by means of TEM. The bar corresponds to 65 nm. C) VLPs sample obtained by means of the IBDV polyprotein expression in mammal cells by means of infection with the VT7/Poly recombinant vaccine virus (Fernández-Arias *et al.*, (1998), cited *supra*). The bar corresponds to 65 nm.

5 **Figure 5. Structural and biochemical characterization of QVLPs-CD8.** Panel A shows a TEM image of a sample stained with uranyl acetate corresponding to fraction 4 of a sucrose gradient used for the purification of structures carried out on an insect cell extract coinfectd with the FB/pVP2 and PF/his-CD8-VP3 rBVs. The bar indicates 100 nm. Panel B shows the SDS-PAGE and Western blot analyses carried out with an antibody against VP3
10 protein, of a sample corresponding to fraction 4 (QVLPs-CD8) of a sucrose gradient used for the purification of structures carried out on an insect cell extract co-infected with the FB/pVP2 and PF/his-CD8-VP3 rBVs (see panel A). A sample of purified IBDV virus (IBDV) was used as a control. The sizes of the molecular mass (MW) markers, as well as the molecular mass estimated for the VP3 and his-CD8-VP3 proteins, were indicated.

15 **Figure 6.- Enhancing effect of the specific anti-malaria CD8 cellular immune response by means of immunization with IBDV CVLPs containing the *Plasmodium yoelii* CD8 epitope.** Groups of 4 mice from the BALB/c strain were intraperitoneally inoculated with 50 µg/mouse of QVLPs-CD8 (group IV) or non-chimeric VLPs (group III). A group was inoculated with VVpJRCS (10^7 pfu/mouse), a recombinant virus expression the
20 whole *Plasmodium yoelii* CHITOSAN protein (group II) as a control. 15 days later, the mice of all the groups were intraperitoneally immunized with VVpJRCS (10^7 pfu/mouse). One of the groups received at that time a single dose of the viral vector (group I). 15 days after the second immunization, the animals were sacrificed, the spleen was removed and the ELISPOT was carried out against the malaria CD8 peptide. Panel A shows the image of the
25 ELISPOT wells carried out with different concentrations of splenocytes obtained from the mice belonging to each one of the groups after their incubation in the presence (+CD8 peptide) or absence (-CD8 peptide) of the CD8 peptide. Panel B shows a graph of the results obtained as a number of specific IFN- γ / 10^6 splenocyte secreting cells.

30 DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the invention provides an chimeric empty capsid of the infectious bursal disease virus (IBDV), hereinafter CVLP of the invention, characterized in that it is constituted by assembly of (i) IBDV pVP2 proteins and (ii) fusion proteins comprising a

region A constituted by the IBDV VP3 protein bound to a region B constituted by a heterologous polypeptide comprising a polypeptide of interest.

The term "IBDV", as it is used in the present invention, refers to the different IBDV strains belonging to any of the known serotypes (1 or 2) [by way of illustration, see the review carried out by van den Berg TP, Eterradossi N, Toquin D, Meulemans G., en *Rev Sci Tech* 2000 19: 509-43] and the terms "IBDV pVP2 protein" and "IBDV VP3 protein" refer to the different forms of the pVP2 and VP3 proteins representative of any of the mentioned IBDV strains [NCBI protein databank], according to the definition made by Sánchez and Rodríguez (1999) (Sánchez AB, Rodríguez JF. Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology*. 1999 Sep 15; 262(1):190-199), as well as proteins substantially homologous to said IBDV pVP2 and VP3 proteins, i.e. proteins the amino acid sequences of which have a degree of identity regarding said IBDV pVP2 and VP3 proteins of at least 60%, preferably of at least 80%, more preferably of at least 90% and even more preferably of at least 95%.

The IBDV pVP2 protein present in the CVLP of the invention can be any pVP2 protein representative of any IBDV strain, for example, the full-length pVP2 protein of IBDV Soroa strain [NCBI, access number AAD30136].

The fusion protein present in the CVLP of the invention comprises a region A constituted by the IBDV VP3 protein bound to a region B constituted by a heterologous polypeptide comprising a polypeptide of interest. In a particular embodiment, said region B is bound to the amino-terminal region or to the carboxy-terminal region of said IBDV VP3 protein.

The IBDV VP3 protein, constituting region A of said fusion protein, can be any VP3 protein representative of any IBDV strain, for example, the full-length VP3 protein of IBDV Soroa strain [NCBI, access number AAD30136].

Region B present in said fusion protein is constituted by a heterologous polypeptide comprising a polypeptide of interest. As it is used in the present invention, the term "heterologous polypeptide" refers to a polypeptide not belonging to the native IBDV capsid. The size of the polypeptide of interest can vary within a broad interval, from a few amino acids up to hundreds of amino acids. Said polypeptide of interest can be virtually any polypeptide, regardless of its origin (eukaryotic, prokaryotic, viral, etc.), susceptible to being expressed in a recombinant manner. However, in a particular embodiment said polypeptide of interest is a polypeptide useful in vaccination, therapy or diagnosis, such as an epitope or

determining antigen capable of inducing an immune response in animals and humans against diseases caused by viruses, bacteria, parasites or any other type of microorganism, or against tumor diseases.

In a particular embodiment, said region B comprises a single polypeptide of interest.
5 However, in another particular embodiment, said region B comprises two or more polypeptides of interest, equal or different, which can be forming tandems.

In a particular embodiment, said fusion protein comprises a region A bound to a single region B. In this case, said region B can be bound to the amino-terminal region of VP3, or alternatively to the carboxy-terminal region of VP3, present in region A. As previously
10 mentioned region B can contain one or more polypeptides of interest. In a particular embodiment, said region B contains a single polypeptide of interest, whereas in another particular embodiment, said region B comprises two or more different polypeptides of interest.

In another particular embodiment, said fusion protein comprises a region A bound to two regions B, one of them bound to the amino-terminal region of VP3 present in region A and
15 the other one to the carboxy-terminal region of VP3 present in region A. Said regions B can be equal or different, and each one of them can contain one or more polypeptides of interest, which can be equal to or different from one another. In a specific embodiment, the fusion protein comprises a region A bound to a first region B containing a first polypeptide of interest (B1) and a second region B containing a second polypeptide of interest (B2). Said polypeptides
20 of interest (B1) and (B2) can be equal or different. In a specific embodiment, said polypeptides of interest (B1) and (B2) are different from one another.

Generally, region A (constituted by the IBDV VP3 protein) is not bound directly to said region B (constituted by the heterologous polypeptide comprising a polypeptide of interest), but rather through a linker polypeptide between said regions A and B. Therefore, if
25 desired the fusion protein of the invention can further contain a linker polypeptide located between said regions A and B. Advantageously, said linker polypeptide is a peptide with structural flexibility, preferably a polypeptide giving rise to a non-structured domain able to induce an immune response or not. By way of illustration, said flexible peptide can contain repetitions of amino acid residues, particular Gly and Ser residues, or any other suitable
30 repetition of amino acid residues.

The CVLPs of the invention can be obtained by means of the simultaneous expression of said IBDV pVP2 proteins and said fusion protein comprising said regions A and B, in suitable host cells. Said suitable host cells are cells containing the encoding

nucleotide sequence of said fusion protein comprising regions A and B, and the encoding nucleotide sequence of the IBDV pVP2 protein, either in a single gene construct or in two gene constructs. In a particular embodiment, said suitable host cells are cells that are transformed, transfected or infected with a suitable expression system, such as an expression system comprising a gene construct, wherein said gene construct comprises the nucleotide sequence encoding for said fusion protein comprising regions A and B, and the nucleotide sequence encoding for the IBDV pVP2 protein, or else alternatively with an expression system comprising a first gene construct comprising the nucleotide sequence encoding for said fusion protein comprising regions A and B, and a second gene construct comprising the nucleotide sequence encoding for the IBDV pVP2 protein.

Therefore, in another aspect, the invention provides a nucleic acid, the nucleotide sequence of which comprises the nucleotide sequence encoding for said fusion protein forming part of the CVLPs of the invention and comprising a region A constituted by the IBDV VP3 protein bound to a region B constituted by a heterologous polypeptide comprising a polypeptide of interest, wherein said regions B bound to the amino-terminal region or to the carboxy-terminal region of said IBDV VP3 protein. Optionally, the nucleic acid provided by this invention can contain the nucleotide sequence encoding for IBDV pVP2 if desired. More specifically, in a particular embodiment, the nucleic acid sequence provided by this invention comprises (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein and (ii) a nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, and optionally if desired, (iii) a nucleotide sequence comprising the open reading frame or encoding regions corresponding to the IBDV pVP2 protein.

In another particular embodiment, the nucleic acid sequence provided by this invention comprises (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein, (ii) a first nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, (ii') a second nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, wherein said nucleotide sequence can be equal to or different from each first nucleotide sequence, and

optionally if desired, (iii) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein. In this case, one of said first or second nucleotide sequence is operatively bound to 5' end of the nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein and the other one is operatively bound to the 3' end of the nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein.

As it is used in this description, the term "open reading frame corresponding to the pVP2 protein" or "open reading frame corresponding to the IBDV VP3 proteins" includes, apart from the nucleotide sequences of said open reading frames, other open reading frames analogous to the same encoding frames of the IBDV pVP2 and VP3 proteins. Likewise, the term "open reading frame of one or more heterologous polypeptides comprising one or more polypeptides", includes any encoding nucleotide sequence of said heterologous polypeptide(s) comprising one or more polypeptides of interest. As it is used herein, the term "analogous" intends to include any nucleotide sequence which can be isolated or constructed on the base of the encoding nucleotide sequence of IBDV pVP2 and VP3, for example by means of the introduction of conservative or non-conservative nucleotide replacements, including the insertion of one or more nucleotides, the addition of one or more nucleotides at any of the ends of the molecule, or the deletion of one or more nucleotides at any end or inside of the sequence. Generally, a nucleotide sequence analogous to another nucleotide sequence is substantially homologous to said nucleotide sequence. In the sense used in this description, the expression "substantially homologous" means that at the nucleotide level, the nucleotide sequences in question have a degree of identity of at least 60%, preferably of at least 80%, more preferably of at least 90%, and even more preferably of at least 95%.

In another aspect, the invention provides a gene construct comprising a nucleic acid provided by this invention, i.e. a nucleic acid the nucleotide sequence of which comprises the nucleotide sequence encoding for said fusion protein comprising regions A and B, and optionally the nucleotide sequence encoding for said IBDV pVP2 protein. More specifically, in a particular embodiment, the gene construct provided by this invention comprises a nucleotide sequence comprising (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein and (ii) a nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, and optionally if desired, (iii)

a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein. In another particular embodiment, the gene construct provided by this invention comprises a nucleotide sequence comprising (i) a nucleotide sequence comprising (i) a nucleotide sequence comprising the open reading frame or encoding region
5 corresponding to the IBDV VP3 protein, (ii) a first nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, (ii') a second nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, wherein said second nucleotide sequence can be equal to or
10 different from said first nucleotide sequence, and optionally if desired (iii) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein. In this case, one of said first or second nucleotide sequences is operatively bound to the 5' end of the nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein, and the other one is operatively
15 bound to the 3' end of the nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein.

In another aspect, the invention provides an expression vector or system selected from:

- a) an expression system comprising a gene construct provided by this invention,
20 operatively bound to transcription, and optionally translation, control elements, wherein said gene construct comprises the nucleotide sequence encoding for said fusion protein comprising regions A and B and the nucleotide sequence encoding for the IBDV pVP2 protein; and
- b) an expression system comprising a first gene construct provided by this
25 invention, operatively bound to transcription, and optionally translation, control elements, wherein said first construct comprises the nucleotide sequence encoding for said fusion protein comprising regions A and B, and a second gene construct operatively bound to transcription, and optionally translation, control elements, comprising the nucleotide sequence encoding for the IBDV
30 pVP2 protein.

In a particular embodiment, the expression system provided by this invention comprises a gene construct comprising (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3, (ii) a nucleotide sequence

comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, and (iii) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein, wherein said gene construct is operatively bound to transcription, and optionally translation, control elements.

In another particular embodiment, the expression system provided by this invention comprises a first gene construct, operatively bound to transcription, and optionally translation, control elements, said first gene construct comprising (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein, and (ii) a nucleotide sequence comprising the open reading frame or encoding region of one or more heterologous polypeptides comprising one or more polypeptides of interest, and a second gene construct, operatively bound to transcription, and optionally translation, control elements, said second gene construct comprising a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein

The transcription, and optionally translation, control elements present in the expression system provided by this invention include promoters, directing the transcription of the nucleotide sequences of interest (pVP2, VP3 and heterologous polypeptide) to which it is operatively linked, and other sequences necessary or suitable for the transcription and its suitable regulation in time and place, for example, start and end signals, cleavage sites, polyadenylation signal, replication origin, transcriptional activators (enhancers), transcriptional silencers (silencers), etc.

Virtually any suitable expression system or vector can be used in the generation of the expression system provided by this invention. By way of illustration, said suitable expression or vector systems can be selected, according to the conditions and needs of each specific case, from plasmids, bacmids, yeast artificial chromosomes (YACs), bacteria artificial chromosomes (BACs), bacteriophage P1-based artificial chromosomes (PACs), cosmids, or viruses, which can further have a heterologous replication origin, for example, bacterial or of yeast, so that it may be amplified in bacteria or yeasts, as well as a marker usable for selecting the transfected cells different from the gene or genes of interest. These expression systems or vectors can be obtained by conventional methods known by persons skilled in the art [Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory] and form part of the present invention. In a particular embodiment, said expression or vector system is a plasmid, such as

a plasmid suitable for transforming yeasts, for example, the plasmid called pESCURA/pVP2-VP3-GFP (Example 2), or a virus, such as a recombinant baculovirus (rBV), for example, the rBV called FBD/pVP2-his-VP3 (Example 1.2), simultaneously expressing both proteins (IBDV pVP2 and his-VP3) in insect cells during the replication cycle, or the rBVs called FB/pVP2 and FB/his-VP3 (Example 1.1) expressing the IBDV pVP2 and his-VP3 proteins, respectively, when coinfecting insect cells, obtaining IBDV CVLPs with the six histidine (6 his) heterologous polypeptide, or the rBVs called FB/pVP2 and FB/his-CD8-VP3 (Example 3) expressing IBDV pVP2 proteins and his-CD8-VP3, respectively, when co-infecting insect cells, forming the capsids called CD8-CVLPs.

In another aspect, the invention provides a host cell containing the encoding nucleotide sequence of said fusion protein comprising regions A and B, and the encoding nucleotide sequence of the IBDV pVP2 protein, either in a single gene construct or in two different gene constructs. In a particular embodiment, said host cell is a host cell that is transformed, transfected or infected with (i) an expression system provided by this invention comprising either a gene construct wherein said gene construct comprises the nucleotide sequence encoding for said IBDV pVP2 protein and the nucleotide sequence encoding for said fusion protein comprising regions A and B, and the encoding nucleotide sequence of the IBDV pVP2 protein, or else alternatively with (ii) an expression system comprising a first construct comprising the nucleotide sequence encoding for said fusion protein comprising regions A and B, and second gene construct comprising the nucleotide sequence encoding for said IBDV pVP2 protein.

In a particular embodiment, the host cell provided by this invention is a host cell that is transformed, transfected or infected with an expression system comprising a gene construct comprising (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein, (ii) a nucleotide sequence comprising the open reading frame or encoding region of a heterologous polypeptide comprising a polypeptide of interest, and (iii) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein, wherein said gene construct is operatively bound to transcription, and optionally translation, control elements.

In another particular embodiment, the host cell provided by this invention is a host cell that is transformed, transfected or infected with (a) a first gene construct, operatively bound to transcription, and optionally translation, control elements, said first gene construct

comprising (i) a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV VP3 protein, and (ii) a nucleotide sequence comprising the open reading frame or encoding region of a heterologous polypeptide comprising a polypeptide of interest, and with (b) a second gene construct, operatively bound to transcription, and optionally translation, control elements, said second gene construct comprising a nucleotide sequence comprising the open reading frame or encoding region corresponding to the IBDV pVP2 protein.

Virtually any host cell susceptible to being transformed, transfected or infected by an expression system provided by this invention can be used, for example, mammal cells, bird cells, insect cells, yeasts, etc; however, in a particular embodiment, said host cell is selected from yeasts and insect cells. Yeasts are suitable due to the simplicity and production cost. Insect cells are suitable when the expression system comprises one or two recombinant baculoviruses (rBV). The use of rBV is advantageous due to biosafety issues related to the host range of the baculoviruses, incapable of replicating in other cell types which are not insect cells.

In a particular embodiment, the invention provides a host cell, such as a yeast, for example, *Saccharomyces cerevisiae*, *Saccharomyces pombe*, etc., transformed with an expression system, such as a plasmid or an expression vector, comprising a gene construct provided by this invention comprising the nucleotide sequence encoding for said fusion protein comprising regions A and B, and the nucleotide sequence encoding for the IBDV pVP2 protein.

In another particular embodiment, the invention provides a host cell, such as an insect cell, infected with an expression system, such as a recombinant baculovirus, comprising a gene construct provided by this invention comprising the nucleotide sequence encoding for said fusion protein comprising regions A and B, and the nucleotide sequence encoding for the IBDV pVP2 protein.

In another particular embodiment, the invention provides a host cell, such as an insect cell, coinfecting with an expression system comprising a first recombinant baculovirus comprising a gene construct provided by this invention comprising the nucleotide sequence encoding for said fusion protein comprising regions A and B, and with a second recombinant baculovirus comprising a gene construct provided by this invention comprising the nucleotide sequence encoding for the IBDV pVP2 protein.

In another aspect, the invention provides a process for the production of CVLPs of the invention comprising culturing a host cell provided by this invention containing the encoding nucleotide sequence of said fusion protein comprising regions A and B, and the encoding nucleotide sequence of IBDV pVP2, either in a single gene construct or in two different gene constructs, and simultaneously expressing said IBDV pVP2 proteins and fusion protein comprising regions A and B, and if desired, recovering said CVLPs of the invention. In a particular embodiment, said host cell provided by this invention is a cell that is transformed, transfected or infected with a suitable expression system, such as an expression system comprising a gene construct, wherein said gene construct comprises the nucleotide sequence encoding for said fusion protein comprising regions A and B, and the nucleotide sequence encoding for IBDV pVP2, or else alternatively with an expression system comprising a first gene construct comprising the nucleotide sequence encoding for said fusion protein comprising regions A and B, and a second gene construct comprising the nucleotide sequence encoding for IBDV pVP2.

Said process therefore comprises the gene coexpression of said IBDV pVP2 proteins and fusion proteins comprising regions A and B as two independent genes. After the simultaneous expression of said proteins (IBDV pVP2 and fusion proteins comprising regions A and B) in said cells, the expressed proteins are assembled and form the CVLPs of the invention, which can be isolated or withdrawn from the medium and purified if desired. The isolation and purification of said CVLPs of the invention can be carried out by conventional methods, for example, by means of fractioning on sucrose gradients.

In a particular embodiment, the simultaneous gene coexpression of IBDV pVP2 proteins and fusion proteins comprising regions A and B is carried out by means of the use of an rBV allowing the simultaneous expression of said proteins from two independent chimeric genes in insect cells. In this case, the process for the production of CVLPs of the invention provided by this invention comprises, first the obtainment of a gene expression system constituted by an rBV containing a gene construct simultaneously encoding for the IBDV pVP2 proteins and for said fusion proteins comprising regions A and B, such as the rBV called FBD/pVP2-his-VP3 (Example 1.2), or alternatively the obtainment of an rBV containing a gene construct encoding for the IBDV pVP2 protein and the obtainment of another rBV containing a gene construct encoding for said fusion protein comprising regions A and B, such as the rBVs called FB/pVP2 and FB/his-VP3 (Example 1.1), or rBVs called FB/pVP2 and FB/his-CD8-VP3 (Example 3), respectively, followed by the infection of insect cells with said

expression system based on said recombinant baculovirus(es), expression of the recombinant proteins and if desired, isolation of the CVLPs of the invention formed by assembly of said IBDV pVP2 proteins and fusion proteins comprising regions A and B, and optionally subsequent purification of said CVLPs of the invention.

- 5 The construction of a recombinant baculovirus allowing the independent expression of the IBDV pVP2 proteins and the fusion proteins comprising regions A and B can be carried out by any person skilled in the art based on that described herein and on the state of the art concerning this technology (Cold Spring Harbor, N.Y.; Leusch MS, Lee SC, Olins PO. 1995. A novel host-vector system for direct selection of recombinant baculoviruses (bacmids) in
10 *Escherichia coli*. Gene 160: 191-4; Luckow VA, Lee SC, Barry GF, Olins PO. 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J Virol 67: 4566-79).

- 15 In another particular embodiment, the gene coexpression of the IBDV pVP2 proteins and of the previously defined fusion proteins comprising regions A and B is carried out by means of the use of a vector allowing the expression of said proteins in yeast cells. In this case, the process for the production of CVLPs of the invention provided by this invention comprises, first, the obtainment of a gene expression system constituted by a plasmid containing a gene construct simultaneously encoding for the IBDV pVP2 proteins and for said fusion proteins
20 comprising regions A and B, followed by the transformation of yeasts with said expression system, expression of the recombinant proteins, and if desired, isolation of the CVLPs of the invention formed by assembly of said IBDV pVP2 proteins and fusion proteins comprising regions A and B, and optionally subsequent purification of said CVLPs of the invention. In a specific embodiment, the suitable expression system for transforming yeasts is based on a
25 pESC Yeast (Stratagene) expression system such as, for example, the plasmid pESCURA/pVP2/VP3-GFP (Example 2) containing a gene construct encoding for the IBDV pVP2 and VP3-GFP proteins.

- 30 The obtainment of yeasts transformed with a gene construct or with a suitable expression system or vector allowing the simultaneous expression of the IBDV pVP2 proteins and the fusion proteins comprising regions A and B can be carried out by any person skilled in the art based on that described herein and on the state of the art concerning this technology (pESC epitope tagging vectors Instructions manual. Stratagene www.stratagene.com;

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory).

In another aspect, the invention is related to the use of the gene expression system provided by this invention for producing and obtaining the CVLPs of the invention.

5 The CVLPs of the invention can be used as vectors or vehicles of products of interest, such as molecules with biological activity, for example, drugs, polypeptides, proteins, nucleic acids, etc., whereby they can be used for therapeutic or diagnostic or research purposes. In a particular embodiment, said molecules of biological interest include polypeptides of interest, such as antigens or immune response inducers in animals or humans to whom they are
10 supplied, or nucleic acid sequences, useful in gene therapy, intended for being introduced inside the suitable cells.

Therefore, in another aspect, the invention is related to the use of the CVLPs of the invention in the manufacture of medicaments, for example vaccines, gene therapy vectors (delivery systems), etc. In a particular embodiment, said medicament is a vaccine intended for
15 conferring protection against human or animal diseases caused by viruses, bacteria, parasites, or any other type of microorganism, or against tumor diseases. In another particular embodiment, said medicament is a gene therapy vector.

In another aspect, the invention provides a vaccine comprising a therapeutically effective amount of CVLPs of the invention, optionally together with one or more
20 pharmaceutically acceptable adjuvants and/or vehicles. Said vaccine is useful for protecting animals and humans against diseases caused by microorganisms (viruses, bacteria, parasites, etc.), or against tumor diseases. In a particular embodiment, said vaccine is especially useful for simultaneously protecting animals or humans against the infection caused by two or more infectious disease-causing agents. By way of illustration, the vaccine provided by this
25 invention can be used to protect birds, for example chickens, turkeys, geese, pheasants, quails, ostriches, etc., against the infectious bursal disease virus (IBDV) and against one or more infectious agents responsible for avian diseases (avian pathogens).

In the sense used in this description, the expression "therapeutically effective amount" refers to the amount of CVLPs of the invention calculated for producing the desired effect and
30 will generally be determined, among others, by the characteristics of the CVLPs and the immunization effect to be achieved.

The pharmaceutically acceptable adjuvants and vehicles which can be used in said vaccines are those adjuvants and vehicles known by the persons skilled in the art and

normally used in the manufacture of vaccines.

In a particular embodiment, said vaccine is prepared in form of an aqueous solution or suspension in a pharmaceutically acceptable diluent, such as saline solution, phosphate-buffered saline solution (PBS), or any other pharmaceutically acceptable diluent.

5 The vaccine provided by this invention can be administered by any suitable administration route which results in a protective immune response against the heterologous sequence or epitope used, to which end said vaccine will be formulated in the dosage form suited to the chosen administration route. In a particular embodiment, the administration of the vaccine provided by this invention is carried out parenterally, for example,
10 intraperitoneally, subcutaneously, etc.

The following Examples illustrate the invention and should not be considered limiting of the scope thereof.

EXAMPLE 1

15 Obtaining IBDV CVLPs in insect cells

1.1 Obtaining IBDV CVLPs, VP2-his-VP3, by means of two independent rBVs in insect cells

The results of a series of experiments designed to analyze the possibility of obtaining
20 IBDV CVLPs from the coexpression of the IBDV pVP2 and VP3 proteins and a heterologous polypeptide from two independent chimeric genes are described in this example. To that end, two recombinant baculoviruses (rBVs) described above, FB/his-VP3 (Kochan, G., González, D. & Rodríguez, J. F. (2003). Characterization of the RNA binding activity of VP3, a major structural protein of IBDV. *Archives of Virology* 148, 723-744) and
25 FB/VPX, herein cited as FB/pVP2, (Martínez-Torrecuadrada, J. L., Castón, J. R., Castro, M., Carrascosa, J. L., Rodríguez, J. F. & Casal, J. I. (2000). Different architectures in the assembly of infectious bursal disease virus capsid proteins expressed in insect cells. *Virology* 278, 322-331) have been used. These rBVs were generated by means of the cloning into suitable vectors of the complementary DNA (cDNA) encoders of the IBDV pVP2 and pVP3
30 proteins. Said cDNAs were obtained by RT-PCR from the A segment of the serotype I IBDV Soroa strain genome a (NCBI access number AAD30136). The rBV FB/his-VP3 expresses a chimeric VP3 protein which at its N-terminal end contains a tandem of six histidines fused to the VP3 sequence (Met754-Glu1012 of the polyprotein) called his-VP3. rBV FB/pVP2 expresses the encoding region of the pVP2 protein (Met1-Ala512).

The analysis of the expression of these pVP2 and his-pVP3 proteins, whether independently or together, was carried out in cell cultures. To carry out these experiments, single layer cell cultures from the insect *Trichloplusia ni* (H5, Invitrogen) were used, which were grown on cover glasses. Said cultures were independently infected with FB/pVP2, FB/his-VP3, or coinfecting with both rBVs. The multiplicity of infection was 5 pfu/cell. The cells were fixed at 48 hours post-infection (h.p.i.), and incubated with rabbit anti-VP2 polyclonal serum and with rat anti-VP3 polyclonal serum (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054). After successive washings, the cover glasses were incubated with goat anti-rat serum conjugated with Alexa 594 and goat anti-rabbit serum conjugated with Alexa 488 (Jackson ImmunoResearch Laboratories, Inc.). The cellular cores were stained with the specific To-Pro-3 marker (Molecular Probes, Inc.). The samples were finally viewed by epifluorescence with a Zeiss Axiovert 200 microscope equipped with the Bio Rad Radiance 2100 confocal system. The images obtained were stored using the Laser Sharp Package (Bio Rad) software equipment. As is shown in Figure 2a, in the cultures infected with FB/pVP2, the anti-VP2 serum showed a fine granular signal mixed with tubular structures, both distributed throughout the cytoplasm. The anti-VP3 signal, detected in the cells infected with rBV FB/his-VP3, was characterized by the presence of spherical-shaped, and apparently hollow, accumulations around the core. In the cultures coinfecting with both rBVs, a notable modification in the distribution pattern of both proteins was detected. In these cells, the specific signals of pVP2 and VP3 were collocated in spherical and dense accumulations, suggesting that their coexpression allowed the formation of pVP2/his-VP3 complexes (Figure 2c to 2e).

For the purpose of characterizing these structures in greater detail, similar extracts corresponding to cells infected with FB/pVP2+FB/hisVP3 were analyzed by transmission electron microscopy (TEM). As a control, and in parallel, H5 cell cultures infected with the wild strain of the FBD (FastBacDual, Invitrogen) virus were analyzed by the same technique. After the infection, the cells were harvested after 48 hours, and processed as has been previously described (Fernández-Arias A, Risco C, Martínez S, Albar JP & Rodríguez JF. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79:1047-1054) for their analysis in ultrathin sections by TRANSMISSION ELECTRON MICROSCOPY. As is shown in Figure

2, the cytoplasm of the coinfecting cells contains aggregates formed by a mixture of tubules and structures similar to capsids (Figure 2g, 2h and 2i). These aggregates were not observed in any case in the samples corresponding to cells infected with wild FBD virus (Figure 2f). The appearance and size of the tubules, as well as of the structures similar to capsids, was similar to those previously described in cell cultures infected with VT7/Poly, a recombinant of the vaccinia virus expressing the gene of the IBDV polyprotein (Fernández-Arias A, Risco C, Martínez S, Albar JP & Rodríguez JF. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79:1047-1054).

10 To unmistakably establish that the coexpression of pVP2 and his-VP3 enabled the assembly and, therefore, the obtainment of CVLPs, the decision was made to purify the formed particles. To that end, H5 cell cultures were infected with FB/pVP2+FB/his-VP3. At 60 h.p.i., the cells were homogenized and the extracts were separated on sucrose gradients as previously described (Lombardo E, Maraver A, Castón JR, Rivera J, Fernández-Arias A, Serrano A, Carrascosa JL & Rodríguez JF. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73:6973-6983). After their centrifugation, the gradients were fractioned, and the different fractions were analyzed by TEM as previously described (Lombardo E, Maraver A, Castón JR, Rivera J, Fernández-Arias A, Serrano A, Carrascosa JL & Rodríguez JF. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73:6973-6983). As a control, and subject to the same process, gradients corresponding to cell extracts infected with rBV FB/VPX or with rBV FBD/Poly-VP1, were fractioned. The recombinant virus FBD/Poly-VP1 simultaneously expresses the VP1 polypeptide and polyprotein. As was predictable, the infection with FBD/Poly-VP1 had a result of an efficient production of VLPs (Maraver A, Oña A, Abaitua F, González D, Clemente R, Diaz-Ruiz A, Castón JR, Pazos F & Rodríguez JF. (2003). The oligomerization domain of VP3, the scaffolding protein of infectious bursal disease virus, plays a critical role for capsid formation. *Journal of Virology* 77:6438-49). On the other hand, the fractions corresponding to the cells infected with FB/VPX only contain tubules of a twisted appearance. The gradients corresponding to cells coinfecting with the rBVs FB/pVP2+FB/his-VP3 contain rigid type I tubules in the fractions near the bottom of the

gradient, and CVLPs in the central and top fractions (Figure 3b). The CVLPs isolated from the cells coinfecting with rBV FB/pVP2+FB/his-VP3 had a diameter of 65-70 nm, as well as a typical polygonal contour, absolutely indistinguishable from the purified VLPs of cultures infected with FBD/Poly-VP1 (Maraver, A., Oña, A., Abaitua, F., González, D., Clemente, R., Diaz-Ruiz, A., Caston, J. R., Pazos, F. & Rodríguez, J. F. (2003). The oligomerization domain of VP3, the scaffolding protein of infectious bursal disease virus, plays a critical role for capsid formation. *Journal of Virology* 77:6438-49) or of the cultures infected with VT7/Poly (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054).

For the purpose of achieving a biochemical characterization of the obtained material, Western blot experiments were carried out in which the different fractions were compared with specific sera against the VP1, pVP2, VP3 and VP4 proteins (Fernández-Arias *et al.* 1998, cited *supra*; Lombardo *et al.*, 2000). Cell extracts infected with IBDV were used as a control. The obtained results are shown in Figure 3d. As was expected, the bands corresponding to the VP1 and VP4 polypeptides were only detected in samples corresponding to cells infected with FBD/Poly-VP1. The patterns corresponding to pVP2/VP3 in samples corresponding to cells infected with FBD/Poly-VP1 or coinfecting with FB/VPX+ FB/his-VP3 were similar, two bands corresponding to pVP2 and VP3, respectively, being detected.

1.2 Obtaining IBDV CVLPs, pVP2-his-VP3, by means of a single rBV in insect cells

Furthermore, the construction of the plasmid pFBD/pVP2-his-VP3 was carried out. The first step of the construction was carried out by means of the cloning of the encoding region of the pVP2 protein into the pFBDual vector (Invitrogen). The DNA fragment corresponding to pVP2 was obtained by means of PCR with the oligonucleotides identified as Oligo I (SEQ ID NO: 1) and Oligo II (SEQ ID NO: 2) using the plasmid pVOTE.2/Poly as a mold (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054). The fragment was purified, subjected to digestion with the BglIII and HindIII enzymes and cloned into the pFBDual vector (Invitrogen) previously digested with the BamHI and HindIII enzymes. The resulting plasmid was called pFBD/pVP2. Then, a DNA fragment containing the open reading frame

corresponding to the VP3 protein was obtained by means of digestion of the plasmid pFB/his-VP3 (Kochan et al., 2003, cited *supra*) with the RsrII enzyme, treatment with Klenow, and subsequent restriction with KpnI. This DNA fragment was purified and cloned into the plasmid pFBD/pVP2 previously digested with the SmaI and KpnI enzymes. The resulting plasmid was called pFBD/pVP2-his-VP3 (SEQ ID NO: 3) and contains the encoding nucleotide sequence of the pVP2 proteins and of the his-pVP3 fusion protein containing a heterologous his 6 sequence (the latter is encoded by the complementary chain to the nucleotides 6734-7585 of SEQ ID NO: 3). The amino acid sequence of the pVP2 protein and of the his-VP3 fusion protein (pVP2-his-VP3) encoded by the nucleotide sequence contained in said plasmid pFBD/pVP2-his-VP3 is shown in SEQ ID NO: 4.

The plasmid pFBD/pVP2-his-VP3 allows obtaining an rBV, called FBD/pVP2-his-VP3, expressing both proteins simultaneously during its replication cycle [<http://invitrogen.com/content/sfs/manuals/bevtest.pdf>].

The results obtained with FBD/pVP2-his-VP3 in insect cells are identical to those obtained by means of the coinfection with rBVs FB/pVP2 and FD/his-VP3, IBDV CVLPs with the heterologous six histidine (6 his) polypeptide being obtained.

EXAMPLE 2

Obtaining IBDV CVLPs, pVP2-VP3-GFP, in yeasts

For the purpose of studying the possibility of obtaining IBDV CVLPs in yeast cultures (*Saccharomyces cerevisiae*) the vector pESCURA/pVP2-VP3-GFP was generated with the heterologous GFP gene bound to the VP3 N-terminal end. The first step in the construction of the vector was carried out by means of the cloning of the encoding region of the pVP2 protein into the vector pESCURAinv. The plasmid pESCURAinv was generated by means of digestion of the vector pRS426 (Stratagene) with the PvuII enzyme and religation of the digestion mixture. The resulting vector, pESCURAinv, contains the multiple cloning region in reversed position with regard to that of parent vector pRS426. The DNA fragment corresponding to the pVP2 protein was obtained by means of PCR with the oligonucleotides called Oligo III (SEQ ID NO: 5) and Oligo IV (SEQ ID NO: 6) using the plasmid pVOTE.2/Poly as a mold (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054). The fragment was purified subjected to digestion with the BglII and HindIII enzymes and cloned

into the vector pESCURA.inv, previously digested with the BamHI and HindIII enzymes. The resulting plasmid was called pESCURA/pVP2.

The plasmid pFB/VP3-GFP was constructed in two stages. The first one consisted of the cloning of a DNA fragment, generated by means of PCR, containing the ORF of the VP3 protein lacking the termination codon. This PCR was carried out using the oligonucleotides called Oligo V (SEQ ID NO: 9) and Oligo VI (SEQ ID NO: 10) and using the plasmid pVOTE.2/Poly as a mold (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054). The resulting DNA was digested with the EcoRI and BamHI enzymes and cloned into the vector pEGFP-N3 (Clontech), also digested with the same enzymes. The resulting plasmid was called pVP3-GFP. Then, the plasmid pEGFP-GFP was digested with the EcoRI and NotI enzymes and cloned into the vector pFastBac1 (Invitrogen). The resulting plasmid was called pFB/VP3-GFP.

Next, a DNA fragment that contained the open reading frame corresponding to the VP3 protein fused to the encoding region of the EGFP protein was obtained by means of digestion of the plasmid pFB/VP3-GFP with the EcoRI and NotI enzymes. This DNA fragment was purified and cloned into the plasmid pESCURA/pVP2 previously digested with the EcoRI and NotI enzymes. The resulting plasmid was called pESCURA/pVP2-VP3-GFP (SEQ ID NO: 7) and contains the ORFs of the pVP2 and VP3-GFP proteins under the transcriptional control of two independent promoters, GAL 1 and GAL 10, both inducible by galactose (the pVP2 protein is encoded by the chain of nucleotides complementary to the nucleotides 5862-7343 of SEQ ID NO: 7). The amino acid sequence of the pVP2 protein and of the VP3-GFP fusion protein (pVP2-VP3-GFP) encoded by the nucleotide sequence contained in said plasmid pESCURA/pVP2-VP3-GFP is shown in SEQ ID NO: 8.

pESCURA/pVP2-VP3-GFP was subsequently used to transform a culture of *S. cerevisiae* yeast haploid strain 499 according to a previously described protocol (Gietz, R.D. and R.A. Woods. (2002), Transformation of yeast by the Liac/SS carrier DNA/PEG method. *Methods in Enzymology* 350:87-96). The yeasts transformed with the plasmid were selected by means of growth on SC medium plates (CSM + YNB, 2% glucose and bacto agar) supplemented with the amino acids tryptophan, leucine and histidine and lacking uracyl (-Ura). After an incubation of 48 hours at 30°C, a colony was chosen which was used to carry out the following protein expression and CVLP formation analyses.

The pVP2 and VP3 protein expression and CVLP formation analyses were carried out following a protocol previously described for the characterization of IBDV VLPs in other expression systems (Fernández-Arias, A., Risco, C., Martínez, S., Albar, J. P. & Rodríguez, J. F. (1998). Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. *Journal of General Virology* 79, 1047-1054; Lombardo, E., Maraver, A., Castón, J. R., Rivera, J., Fernández-Arias, A., Serrano, A., Carrascosa, J. L. & Rodríguez, J. F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73, 6973-6983). The colony selected was cultured in liquid CSM (-Ura) + YNB medium supplemented with 2% raffinose. The culture was incubated at 30°C for 24 hours. This culture was used to inoculate, at an optical density (O.D.) of 0.2, a flask of 200 ml of CSM (-Ura) + YNB medium supplemented with 2% inducer galactose. The culture was maintained at 30°C for 18 hours (until an O.D. between 1.0 and 2.0). The yeasts were centrifuged at 3,000 *g* for 5 minutes at 4°C, were washed once with distilled water, and the pellet was resuspended in lysis buffer (TEN: Tris 10 mM, pH 8.0; NaCl 150 mM; EDTA 1 mM) + 2X protease inhibitors (Compl Roche). A volume of glass beads having a size of about 425-600 microns (Sigma) were added for the lysis. This mixture was subjected to vigorous vortex stirring for 30 seconds 4 times, with 30-second intervals, and at 4°C. After this, the soluble fraction was recovered by centrifuging the lysis mixture at 13,000 rpm for 15 minutes at 4°C. This sample was subjected to fractioning on a sucrose gradient according to a previously described protocol (Lombardo, E., Maraver, A., Castón, J. R., Rivera, J., Fernández-Arias, A., Serrano, A., Carrascosa, J. L. & Rodríguez, J. F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73, 6973-6983). The samples obtained after fractioning as well as a sample of the starting material were analyzed by means of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [Current Protocols in Molecular Biology] and immunodetection by Western blot (Figure 4A) using anti-pVP2 and anti-VP3 sera [Current Protocols in Molecular Biology]. As is shown in Figure 4A, the Western blot showed the presence of bands, with the predicted molecular mass corresponding to the pVP2 (48 kDa) and VP3-GFP (61 kDa) proteins, as well as other immunoreactive bands of a smaller size probably produced by proteolytic degradation both in the initial sample and in the different

fractions of the gradient. These results reliably showed the correct expression of both polypeptides in the *S. cerevisiae* culture transformed with the plasmid pESCURA/pVP2-VP3. Then, the different fractions of the gradient were analyzed by means of TEM as has been previously described (Lombardo, E., Maraver, A., Castón, J. R., Rivera, J., Fernández-Arias, A., Serrano, A., Carrascosa, J. L. & Rodríguez, J. F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73, 6973-6983). As is shown in Figure 4B, the TEM analysis of the fractions of the gradient showed the existence of IBDV CVLPs in the top fractions of the gradient. These CVLPs have a diameter of 65-70 nm and a polygonal contour that is indistinguishable from the IBDV CVLPs obtained in other expression systems (Figure 4C).

EXAMPLE 3

Obtaining and characterizing the immunogenicity of IBDV CVLPs

As part of the development of new vaccination strategies, the possibility of using the strategy of producing chimeric IBDV VLPs (CVLPs) which contained heterologous amino acid sequences corresponding to other proteins or peptides involved in the induction of an immune response was analyzed. As a study model, the possibility of obtaining CVLPs which contained, as a heterologous polypeptide comprising a polypeptide of interest, the amino acid sequence corresponding to the CD8 epitope (E-CD8) of the malaria CS protein (*Plasmodium yoelii*), was approached. (Quantification of antigen specific CD8+ T cells using an ELISPOT assay. J Immunol Methods 181: 45-54; Zavala, F., Rodrigues, M., Rodriguez, D., Rodriguez, J. R., Nussenzweig, R. S. and Esteban, M. (2001). A striking property of recombinant poxviruses: efficient inducers of in vivo expansion of primed CD8(+) T cells. *Virology* 280: 155-159). This epitope is responsible for the CD8-specific cellular immune response induction against this pathogen (Oliveira-Ferreira J, Miyahira Y, Layton GT, Savage N, Esteban M, Rodriguez D, Rodriguez JR, Nussenzweig RS, Zavala F, Myahira Y. (2000). Immunogenicity of Ty-VLP bearing a CD8(+) T cell epitope of the CS protein of *P. yoelii*: enhanced memory response by boosting with recombinant vaccinia virus. *Vaccine* 18: 1863-1869). This response can be quantified by means of the ELISPOT technique (Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM, Zavala F. (1995) Quantification of antigen specific CD8+ T cells using an ELISPOT assay. J Immunol Methods 181: 45-54) in splenocyte cultures from BALB/c mice.

For this purpose, the construction of the plasmid pFB/his-CD8-VP3 (SEQ ID NO: 13) was carried out following the cloning strategy described later. This vector was constructed by means of insertion of a 36 bearing portion DNA fragment, generated by means of hybridization of the synthetic oligonucleotides identified as CD8 A (SEQ ID NO: 11) and CD8 B (SEQ ID NO: 12), containing the encoding sequence of the CD8 epitope (SYVPSAEQI, see residues 29 to 37 of the SEQ ID NO: 13 and 14) of the malaria CS protein in the ORF encoding the his-VP3 protein integrated in the pFB/his-VP3 vector. The cloning was carried out by means of ligation of the DNA fragment generated by means of hybridization of the synthetic oligonucleotides CD8 A and B (SEQ ID NO: 11 and SEQ ID NO: 12) to the plasmid pFB/his-VP3 digested with the EheI restriction enzyme. This plasmid pFB/his-CD8-VP3 (SEQ ID NO: 13) contains an ORF encoding a fusion protein called his-CD8-VP3 containing the CD8 epitope inserted in the end corresponding to the N-terminal sequence of the his-VP3 protein ORF. The amino acid sequence of the his-CD8-VP3 fusion protein encoded by the nucleotide sequence contained in said plasmid pFB/his-CD8-VP3 is shown in SEQ ID NO: 14.

The plasmid pFB/his-CD8-VP3 was purified and used to generate the corresponding recombinant baculovirus (rBV), called FB/his-CD8-VP3, following the Bac-to-Bac technology according to the protocols described by the manufacturer (Invitrogen BV, Groningen, The Netherlands).

3.1 Producing CVLPs

H5 cell cultures were simultaneously infected with the recombinant baculoviruses FB/His-CD8-VP3 and FB/pVP2. The FB/pVP2 rBV (see Example 1.1) expresses the region corresponding to the pVP2 protein (Met1-Ala 512) of the IBDV polyprotein. The cells were harvested at 48 hours post-infection (pi), and the corresponding extracts were subjected to the IBDV VLPs purification protocol by means of fractioning on linear sucrose gradients (Lombardo, E., Maraver, A., Castón, J. R., Rivera, J., Fernández-Arias, A., Serrano, A., Carrascosa, J. L. & Rodríguez, J. F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of Virology* 73: 6973-6983). Each one of the obtained fractions was viewed by means of transmission electron microscopy (TEM) and analyzed by means of SDS-PAGE and immunoblot using VP3 specific antibody. As is observed in Figure 5A, fraction 4 of the gradient contained abundant assemblies with an identical structure (polygonal perimeter and a diameter of 65-70 nm) as the IBDV VLPs

obtained by means of expression of the viral polyprotein. The biochemical characterization, by means of SDS-PAGE and Western blot (Figure 5B), showed that these CVLPs contain a protein, immunoreactive against the anti-VP3 serum, the molecular mass (33.5 kDa) of which is identical to the aforementioned one for the his-CD8-VP3 fusion protein (33.591 kDa). These results allow concluding that the coexpression of the pVP2 and his-CD8-VP3 genes in insect cells gives rise to the formation of chimeric VLPs (CVLPs) containing the his-CD8-VP3 fusion protein. These CVLPs are called CD8-CVLPs.

3.2 Immunogenicity analysis of CD8-CVLPs

For the purpose of determining the immunogenic capacity of the CD8-CVLPs two identical assays were carried out using two batches of CD8-CVLPs independently produced and purified. Four groups (I, II, III and IV) of three female eight-week old BalbC rats were used. The groups were formed randomly. The immunization strategy was similar to the one used previously in the characterization of other immunogens. This strategy is based on the use of a priming dose with the antigen under study, followed by a second booster dose, which amplifies the primary response, with the recombinant vaccinia virus VVpJRCS, which expresses the malaria CS protein. The induced immune response was determined by means of the detection of the antigen specific CD8⁺ T cells according to their ability to produce IFN- γ , by means of an ELISPOT assay (Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM, Zavala F. (1995). Quantification of antigen specific CD8⁺ T cells using an ELISPOT assay. J Immunol Methods 181: 45-54). In summary, 96-well plates with nitrocellulose (Millipore) bottoms were coated with 75 μ l/well of a solution containing 6 μ g/ml of the rat anti-murine IFN- γ monoclonal antibody (R4-6A2, Pharmingen, San Diego, CA) resuspended in PBS. The plates were incubated overnight at room temperature. The wells were subsequently washed three times with RPMI medium, and were finally incubated with RPMI medium supplemented with 10% fetal calf serum (FCS) for one hour at 37°C 5% CO₂ atmosphere. On the other hand, the spleens of the immunized rats, maintained in RPMI medium supplemented with 10% FCS, were arranged on a sterile grid on a 60 movable member plate and were homogenized, the extract breaking up by means of its passing through needles of different gauges (21G->25G). The cells thus broken up were centrifuged for 5 minutes at 1,500 rpm at 4°C, and were washed twice with RPMI + 10% FCS medium. In order to lyse the erythrocytes of the samples, sterile 0.1 M NH₄Cl (2 ml/spleen) was added and it was maintained at 4°C for 3-5 minutes, RPMI + 10% FCS was added and it was centrifuged.

Then, they were twice and it was finally resuspended in 1-2 ml RPMI + 10% FCS. The splenocyte viability count was carried out by means of trypan blue staining (4% in water, Sigma).

The professional antigen-presenting cells (APCs) used in this assay were P815. These cells were adjusted to a concentration of 10^6 cells/ml and were incubated with the synthetic peptide SYVPSAEQI (corresponding to the CD8 region of the malaria CS protein) 10^{-6} M. After treatment with the peptide, the cells were washed and treated with mitomycin C (30 μ g/ml) (Sigma) for 15 minutes at 37°C and in CO₂ atmosphere. After subsequent washings, the antigen-presenting cells, to which 30 U/ml of murine interleukin 2 (IL-2) were added, were added at a concentration of 10^5 cells/well. 100 μ l/well of 10^6 splenocytes/ml and 1/4 and 1/16 dilutions were also added. The plates were incubated for 18 ours at 37°C in CO₂ atmosphere, they were washed 5 times with PBST and incubated with 2 μ g/ml of the biotinylated rat anti-IFN- γ XMG1.2 monoclonal antibody (Pharmingen) diluted in PBST for 2 hours at room temperature. Then the plates were washed five times with PBST and a dilution of 1/800 avidin-peroxidase was added (0.5 mg/ml) (Sigma). After 1 hour of incubation at room temperature, it was washed 3 times with PBST and 2 times with PBS, finally adding the developer mixture with 1 μ g/ml of the DAB substrate (Sigma), resuspended in Tris-HCl, pH 7.5, 50 mM, containing 0.015% H₂O₂. The reaction was stopped by washing the plate with abundant water, and once dried, the spots were counted with the aid of a Leica MZ122 APO stereomicroscope and the QWIN Imaging System software (Leica, Cambridge, United kingdom).

Immunizations were carried out according to the immunization program described in the following table:

| Group | 1 st Immunization. Day 0 | 2 nd Immunization. Day 14 |
|-------|-------------------------------------|--------------------------------------|
| I | Not immunized | VVpJRPyCS |
| II | VVpJRPyCS | VVpJRPyCS |
| III | IBDV VLPs | VVpJRPyCS |
| IV | CD8-CVLPs | VVpJRPyCS |

Immunizations with VVpJRCS were carried out intraperitoneally using 10^7 plaque forming units (pfu) per anima. Immunizations with VLPs, both non-chimeric IBDV VLPs and CD8-CVLPs, were carried out intraperitoneally with a dose of 50 μ g of antigen per animal. In all cases, the antigen preparations were diluted in phosphate-buffered saline (PBS).

28 days after the first immunization, the animals were sacrificed and there spleens were used to carry out the ELISPOT assays. These assays were carried out following the protocol described above. Virtually identical results were obtained in both assays. Figure 6 shows the results corresponding to the first assay. The obtained results demonstrate that when CD8-CVLPs are used as a priming dose followed by a booster dose with the VVpJRCS virus (group IV), a strong stimulation of the specific cellular immune response against the malaria CD8 epitope occurs. This stimulation is much greater (about 20 times greater) than that obtained after the immunization with one (group I) or two (group II) doses of VVpJRCS. The fact that a significant stimulation of the response against E-CD8 dose not occur in animals immunized with non-chimeric IBDV VLPs (group III), with regard to group I, which received a single dose of VVpJRCS, demonstrates that the response obtained in group IV is specifically induced by the E-CD8 present in the his-CD8-VP3 fusion protein forming an integral part of the CD8-CVLPs.

CLAIMS

1. A chimeric empty capsid of the infectious bursal disease virus (IBDV), characterized in that it is constituted by assembly of (i) IBDV pVP2 proteins and (ii) fusion proteins comprising a region A constituted by the IBDV VP3 protein bound to a region B constituted by a heterologous polypeptide comprising a polypeptide of interest.
2. Capsid according to claim 1, wherein said region B is bound to the amino-terminal region of IBDV VP3, or alternatively to the carboxy-terminal region of IBDV VP3.
3. Capsid according to claim 1, wherein said polypeptide of interest is a polypeptide useful in vaccination, therapy or diagnosis.
4. Capsid according to claim 1, wherein said region B comprises a single polypeptide of interest.
5. Capsid according to claim 1, wherein said region B comprises two or more polypeptides of interest.
6. Capsid according to claim 1, wherein said fusion protein comprises a region A bound to a single region B.
7. Capsid according to claim 1, wherein said fusion protein comprises a region A bound to two regions B, equal or different, one of them bound to the amino-terminal region of VP3 present in region A, and the other one to the carboxy-terminal region of VP3 present in region A.
8. Capsid according to claim 7, wherein said regions B contain more than one polypeptides of interest equal to or different from one another.
9. Capsid according to claim 1, wherein said fusion protein further comprises, a linker polypeptide located between said regions A and B.

10. A nucleic acid, said nucleic acid having a nucleotide sequence which comprises the nucleotide sequence encoding for the fusion protein defined in anyone of claims 1 to 9.

11. A nucleic acid, said nucleic acid having a nucleotide sequence which comprises
5 (i) a nucleotide sequence comprising the open reading frame corresponding to the IBDV VP3 protein and (ii) a nucleotide sequence comprising the open reading frame of one or more heterologous polypeptides comprising one or more polypeptides of interest.

12. Nucleic acid according to claim 11, further comprising (iii) a nucleotide sequence
10 comprising the open reading frame corresponding to the IBDV pVP2 protein.

13. A gene construct comprising a nucleic acid according to claim 10 or 11.

14. A gene construct comprising a nucleic acid according to claim 12.
15

15. An expression system selected from:

a) an expression system comprising a first gene construct according to claim 13, operatively bound to transcription, and optionally translation, control elements, and a second gene construct, operatively bound to transcription, and optionally translation, control
20 elements; said second gene construct comprising a nucleotide sequence comprising the open reading frame corresponding to the IBDV pVP2 protein; and

b) an expression system comprising a gene construct according to claim 14, operatively bound to transcription, and optionally translation, control elements.

25 16. Expression system according to claim 15, said expression system being selected from plasmids, bacmids, yeast artificial chromosomes (YACs), bacteria artificial chromosomes (BACs), bacteriophage P1-based artificial chromosomes (PACs), cosmids, or viruses, which optionally contain a heterologous replication origin.

30 17. A host cell containing a nucleic acid according to anyone of claims 10 to 12, or a gene construct according to anyone of claims 13 or 14, or an expression system according to anyone of claims 15 or 16.

18. A host cell, said cell having been transformed, transfected or infected with an expression system according to any of claims 15 or 16.

19. Host cell according to claim 17 or 18, said cell being selected from a mammal
5 cell, an avian cell, an insect cell and a yeast.

20. A process for the production of chimeric empty capsids of the infectious bursal disease virus (IBDV) according to anyone of claims 1 to 9, comprising culturing a host cell according to anyone of claims 17 to 19, and, if desired, recovering said chimeric empty
10 IBDV capsids.

21. Process according to claim 20, wherein said host cell is an insect cell, comprising the steps of:

- 15 a) preparing an expression system selected from (I) and (II), wherein:
- expression system (I) is constituted by a recombinant baculovirus containing a gene construct according to claim 14; and
 - 20 - expression system (II) is constituted by a first recombinant baculovirus containing a gene construct encoding for the IBDV pVP2 protein, and a second recombinant baculovirus containing a gene construct according to claim 13;
- 25 b) infecting insect cells with said expression system prepared in step a);
- c) culturing the infected insect cells obtained in step b) under conditions allowing the expression of recombinant proteins and their assembly to form chimeric empty IBDV capsids; and
- 30 d) if desired, isolating and optionally purifying the chimeric empty IBDV capsids.

22. A process according to claim 20, wherein said host cell is a yeast, comprising the steps of:

5. a) preparing an expression system constituted by a plasmid containing a gene construct according to claim 14;
- b) transforming yeast cells with said expression system prepared in step a);
- 10 c) culturing the transformed yeasts obtained in step b) under conditions allowing the expression of recombinant proteins and their assembly to form chimeric empty IBDV capsids; and
- d) if desired, isolating and optionally purifying the chimeric empty IBDV capsids.

15 23. The use of a gene expression system according to anyone of claims 15 or 16 for producing chimeric empty IBDV capsids according to anyone of claims 1 to 9.

20 24. The use of chimeric empty capsids of the infectious bursal disease virus (IBDV) according to anyone of claims 1 to 9 in the manufacture of a medicament.

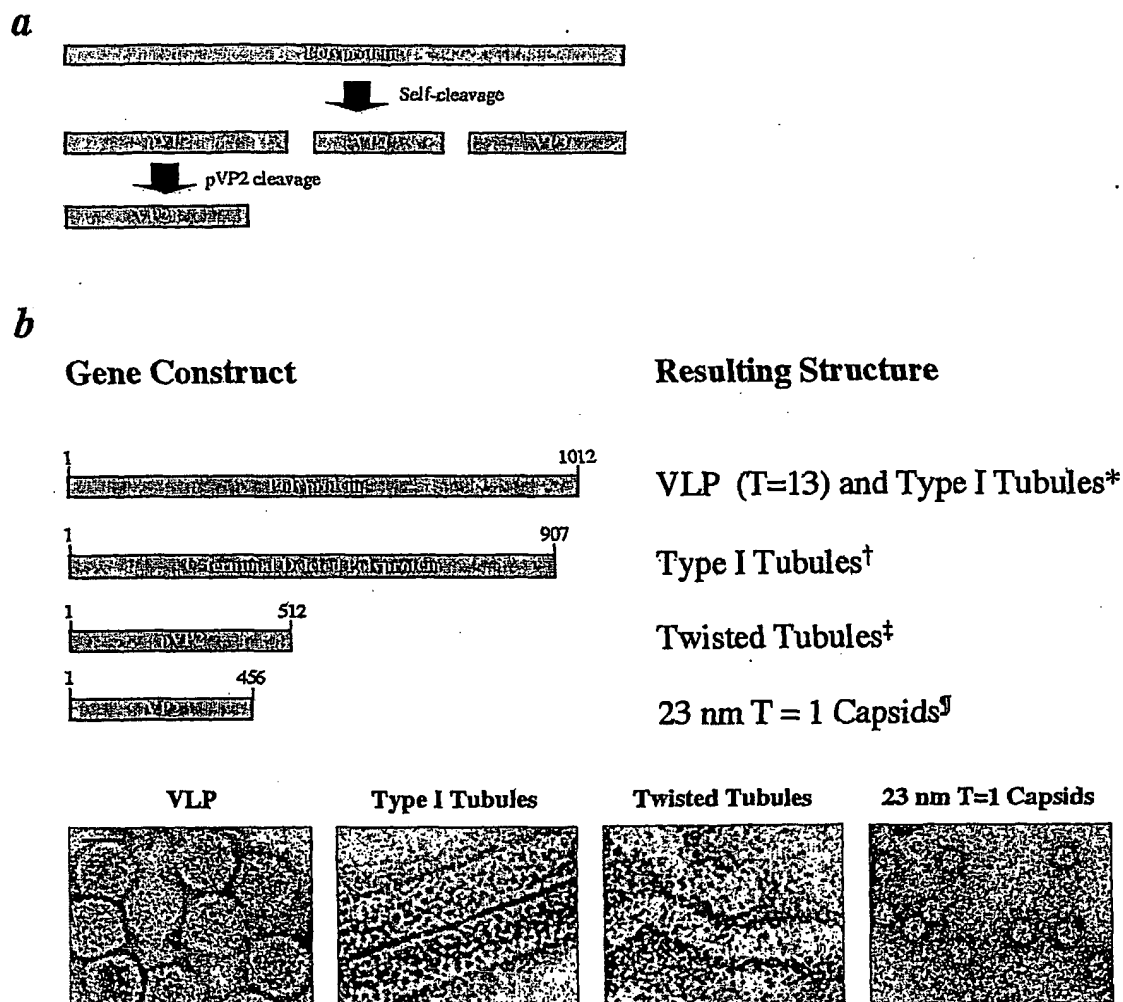
25. Use according to claim 24, wherein said medicament is a vaccine.

26. Use according to claim 24, wherein said medicament is a gene therapy vector.

25 27. A vaccine comprising a therapeutically effective amount of chimeric empty capsids of the infectious bursal disease virus (IBDV) according to anyone of claims 1 to 9, optionally together with one or more pharmaceutically acceptable adjuvants and/or vehicles.

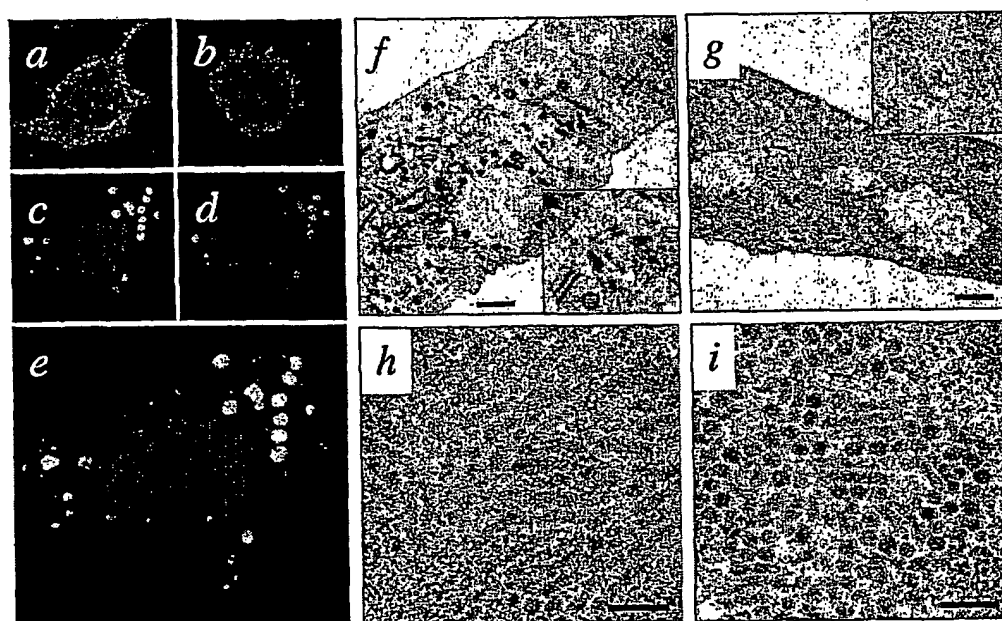
30 28. A vaccine according to claim 27, useful to simultaneously protect animals or humans against infection caused by two or more disease-causing infectious agents.

29. A gene therapy vector comprising a chimeric empty capsid of the infectious bursal disease virus (IBDV) according to anyone of claims 1 to 9.



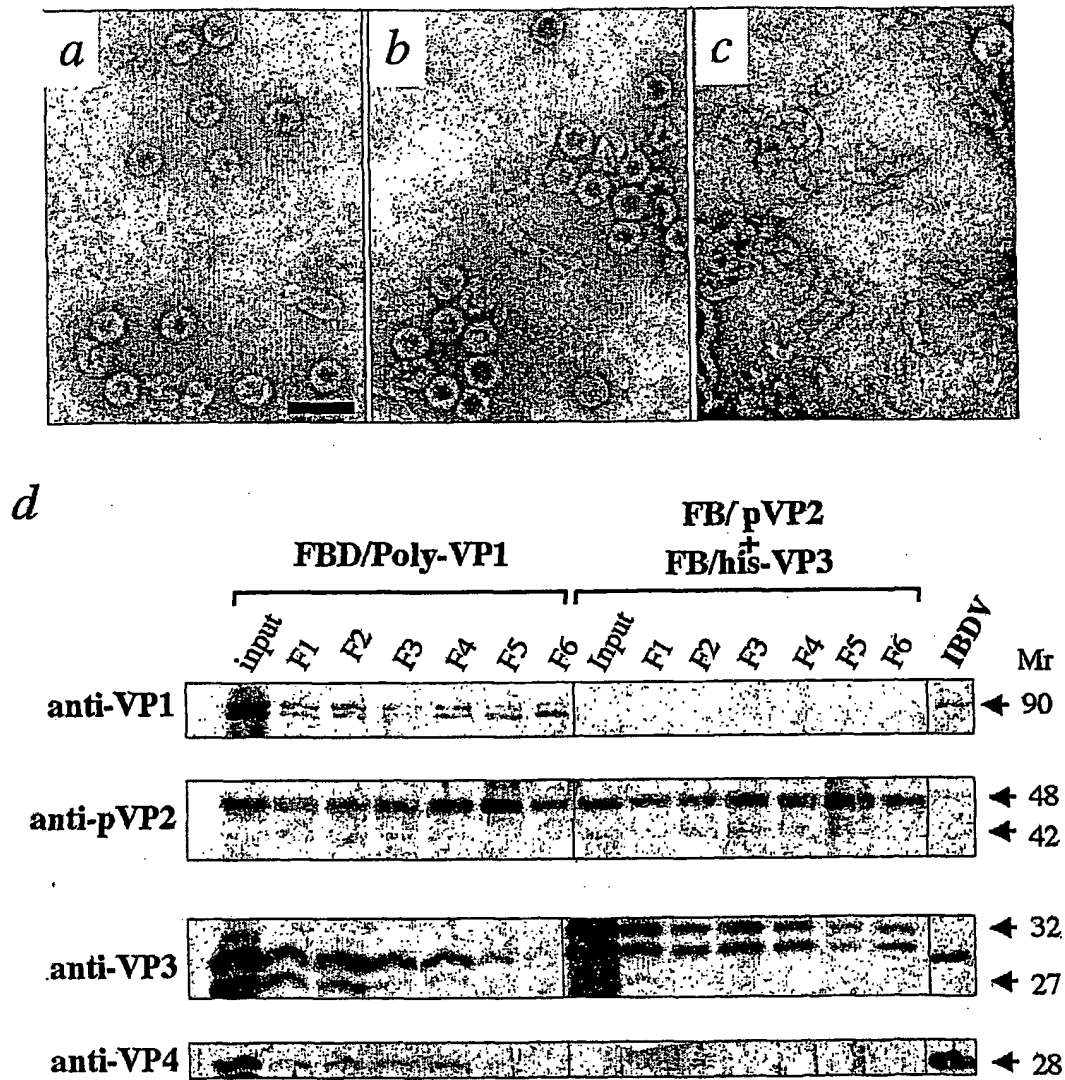
BEST AVAILABLE COPY

Fig. 1



BEST AVAILABLE COPY

Fig. 2



NOT AVAILABLE COPY

Fig. 3

4/6

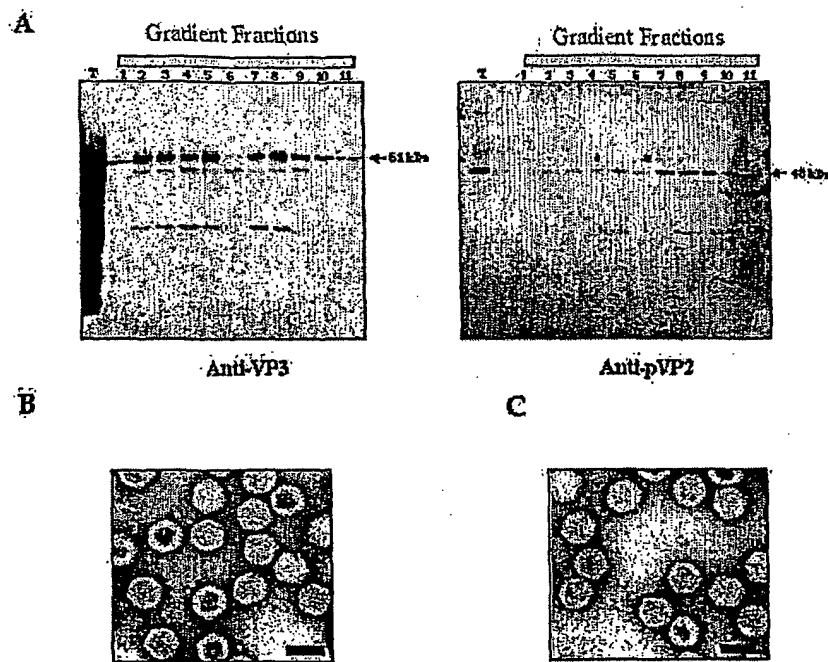


Fig. 4

BEST AVAILABLE COPY

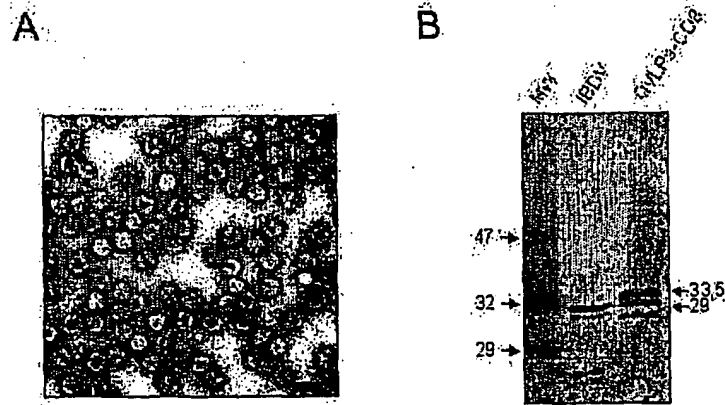


Fig. 5

NOT AVAILABLE COPY

6/6

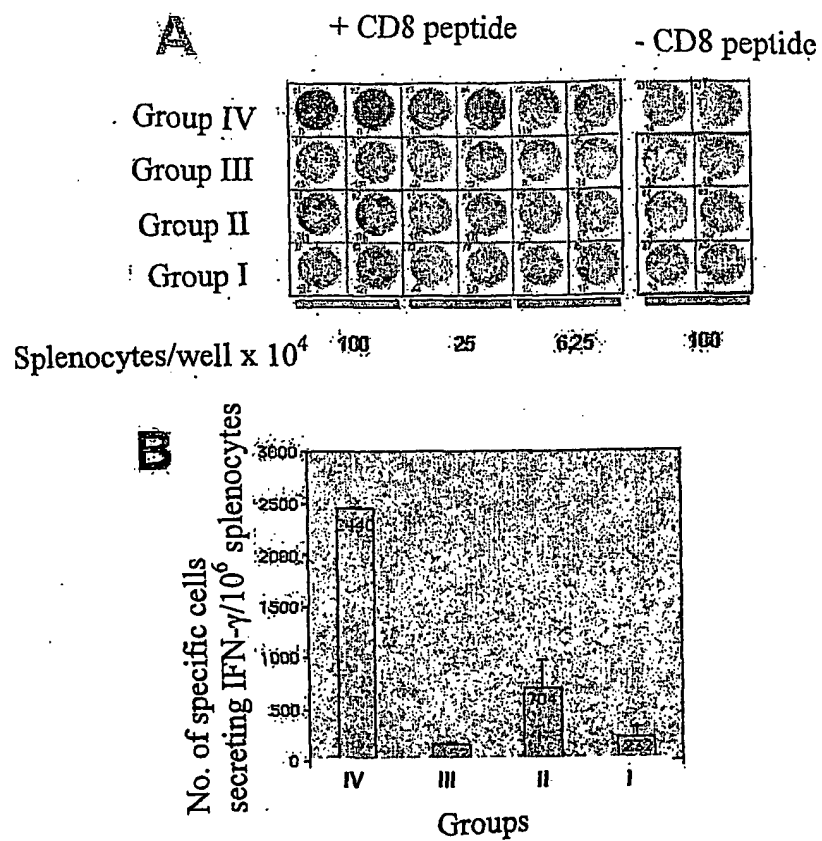


Fig. 6

-201 AVAILABLE COPY

SEQUENCE LISTING

<110> CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS
<110> BIONOSTRA, S.L.

<120> CHIMERIC EMPTY CAPSIDS OF THE INFECTIOUS BURSAL DISEASE VIRUS
(IBDV), OBTAINMENT PROCESS AND APPLICATIONS

<130> P1391PC

<150> ES P200400120
<151> 2004-01-21 (January 21, 2004)

<160> 14

<170> PatentIn version 3.1

<210> 1
<211> 35
<212> DNA
<213> Artificial sequence

<220> Synthetic DNA
<223> Oligo I primer

<400> 1
gcgcagatct atgacaaacc tgtcagatca aaccc 35

<210> 2
<211> 34
<212> DNA
<213> Artificial sequence

<220> Synthetic DNA
<223> Oligo II primer

<400> 2
gcgcaagctt aggcgagagt cagctgcctt atgc 34

<210> 3
<211> 7595
<212> DNA
<213> Artificial sequence

<220>
<223> Plasmid pFBD/pVP2-his-VP3

<220>
<221> promoter
<222> (157)..(285)
<223> Promoter ppolh

<220>
<221> CDS
<222> (291)..(1289)
<223> pVP2 ORF

| | |
|---|------|
| ggg gtc acc gtc ctc agc tta ccc aca tca tat gat ctt ggg tat gtg Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly Tyr Val 165 170 175 | 824 |
| agg ctt ggt gac ccc att ccc gca ata ggg ctt gac cca aaa atg gta Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys Met Val 180 185 190 | 872 |
| gcc aca tgt gac agc agt gac agg ccc aga gtc tac acc ata act gca Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile Thr Ala 195 200 205 210 | 920 |
| gcc gat gat tac caa ttc tca tca cag tac caa cca ggt ggg gta aca Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly Val Thr 215 220 225 | 968 |
| atc aca ctg ttc tca gcc aac att gat gcc atc aca agc ctc agc gtt Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu Ser Val 230 235 240 | 1016 |
| ggg gga gag ctc gtg ttt cga aca agc gtc cac ggc ctt gta ctg ggc Gly Gly Glu Leu Val Phe Arg Thr Ser Val His Gly Leu Val Leu Gly 245 250 255 | 1064 |
| gcc acc atc tac ctc ata ggc ttt gat ggg aca acg gta atc acc agg Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile Thr Arg 260 265 270 | 1112 |
| gct gtg gcc gca aac aat ggg ctg acg acc ggc acc gac aac ctt atg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn Leu Met 275 280 285 290 | 1160 |
| cca ttc aat ctt gtg att cca aca aac gag ata acc cag cca atc aca Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro Ile Thr 295 300 305 | 1208 |
| tcc atc aaa ctg gag ata gtg acc tcc aaa agt ggt ggt cag gca ggg Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln Ala Gly 310 315 320 | 1256 |
| gat cag atg tca tgg tcg gca aga ggg agc cta gcagtgaaga tccatggtgg Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu 325 330 | 1309 |
| caactatcca ggggccctcc gtcccgtcac gctagtggcc tacgaaagag tggcaacagg | 1369 |
| atccgtcggt acggtcgctg gggtagcaa cttcgagctg atcccaaata ctgaactagc | 1429 |
| aaagaacctg gttacagaat acggccgatt tgaccagga gccatgaact acacaaaatt | 1489 |
| gatactgagt gagagggacc gtcttggcat caagaccgtc tggccaacaa gggagtacac | 1549 |
| tgactttcgt gaatacttca tggaggtggc cgacctcaac tctcccctga agattgcagg | 1609 |
| agcattcggc ttcaaagaca taatccgggc cataaggagg atagctgtgc cggtggtctc | 1669 |
| cacattgttc ccacctgccg ctcccctagc ccatgcaatt ggggaaggtg tagactacct | 1729 |
| gctgggcgat gaggcccagg ccgcttcagg aactgctcga gccgcgtcag gaaaagcaag | 1789 |

agctgcctca ggccgcataa ggcagctgac tctcgccctaa gcttgctgag aagtactaga 1849
ggatcataat cagccatacc acatttgtag aggttttact tgcttttaaa aacctccac 1909
aactccccct gaacctgaaa cataaaatga atgcaattgt tgttggttaac ttgtttattg 1969
cagcttataa tggttacaaa taaagcaata gcatacaaaa tttcacaat aaagcatttt 2029
tttcaactgca ttctagttgt ggtttgtcca aactcatcaa tgtatcttat catgtctgga 2089
tctgatcact gcttgagcct aggagatccg aaccagataa gtgaaatcta gttccaaact 2149
at ttgtcat ttttaatttt cgtattagct taogacgcta caccagttc ccatctattt 2209
tgtcaactctt ccctaaataa tcttaaaaa ctccatttcc acccctccca gttcccaact 2269
at ttgtccg ccacagcgg ggcatttttc ttctgttat gtttttaatc aaacatcctg 2329
ccaactccat gtgacaaacc gtcactctcg gctacttttt ctctgtcaca gaatgaaaat 2389
ttttctgtca tctcttcgtt attaatgttt gtaattgact gaatatcaac gcttatttgc 2449
agcctgaatg gcgaatggga cgcgcctgt agcggcgcat taagcgcggc ggggtgtggtg 2509
gttacgcgca gcgtgaccgc tacacttgcc agcgcctag cgcgcgtcc ttctgctttc 2569
ttcccttctt ttctcgccac gttcgccggc tttcccgctc aagctctaaa tggggggctc 2629
cctttagggt tccgatttag tgctttacgg cacctcgacc ccaaaaaact tgattagggt 2689
gatggttcac gtagtgggccc atcgccctga tagacggttt ttcgcccttt gacgttgagg 2749
tccacgttct ttaatagtgg actcttggtc caaactggaa caaactcaa cctatctcg 2809
gtctattctt ttgatttata agggattttg cggatttcgg cctattgggt aaaaaatgag 2869
ctgatttaac aaaaatttaa cgcgaatttt aacaaaatat taacgtttac aatttcaggt 2929
ggcacttttc ggggaaatgt gcgcggaacc cctatttggt tatttttcta aatacattca 2989
aatatgtatc cgctcatgag acaataaccc tgataaatgc ttcaataata ttgaaaaagg 3049
aagagtatga gtattcaaca ttccgtgtc gcccttattc ctttttttgc ggcattttgc 3109
cttctgttt ttgctacccc agaaacgctg gtgaaagtaa aagatgctga agatcagttg 3169
ggtgcacgag tgggttacat cgaactggat ctcaacagcg gtaagatcct tgagagtttt 3229
cgccccgaag aacgttttcc aatgatgagc acttttaaag ttctgctatg tggcgcggt 3289
ttatccgta ttgacgccgg gcaagagcaa ctcggtcgcc gcatacacta ttctcagaat 3349
gacttggttg agtactcacc agtcacagaa aagcatctta cggatggcat gacagtaaga 3409
gaattatgca gtgctgccat aaccatgagt gataaactg cggccaactt acttctgaca 3469
acgatcggag gaccgaagga gctaaccgct tttttgcaca acatggggga tcatgtaact 3529
cgcttgatc gttgggaacc ggagctgaat gaagccatac caaacgacga gcgtgacacc 3589

acgatgcctg tagcaatggc aacaacgttg cgcaactat taactggcga actacttact 3649
ctagcttccc ggcaacaatt aatagactgg atggaggcgg ataaagttgc aggaccactt 3709
ctgcgctcgg cccttcgggc tggctggttt attgctgata aatctggagc cggtagcgt 3769
gggtctcgg gtatcattgc agcactgggg ccagatggta agccctcccg tatcgtagtt 3829
atctacacga cggggagtca ggcaactatg gatgaacgaa atagacagat cgtgagata 3889
ggtgcctcac tgattaagca ttggtaactg tcagaccaag tttactcata tatactttag 3949
attgatttaa aacttcattt ttaatttaaa aggatctagg tgaagatcct ttttgataat 4009
ctcatgacca aaatccotta acgtgagttt tcgttccact gagcgtcaga ccccgtagaa 4069
aagatcaaag gatcttcttg agatcctttt tttctgcgcg taatctgctg cttgcaaaca 4129
aaaaaaccac cgctaccagc ggtggtttgt ttgccggatc aagagctacc aactcttttt 4189
ccgaaggtaa ctggcttcag cagagcgcag ataccaaata ctgtccttct agttagccg 4249
tagttaggcc accacttcaa gaactctgta gcaccgccta catacctcgc tctgctaatac 4309
ctgttaccag tggtgctgc cagtggcgat aagtcgtgtc ttaccgggtt ggactcaaga 4369
cgatagttac cggataaggc gcagcggtcg ggctgaacgg ggggttcgtg cacacagccc 4429
agcttggagc gaacgacctc caccgaactg agatacctac agcgtgagca ttgagaaagc 4489
gccacgcttc ccgaaggag aaaggcggac aggtatccgg taagcggcag ggtcggaaaca 4549
ggagagcgcga cgaggagct tccaggggga aacgcctggt atctttatag tcctgtcggg 4609
tttcgccacc tctgacttga gcgtcgattt ttgtgatgtc cgtcaggggg gcggagccta 4669
tggaanaacg ccagcaacgc ggctttttta cggttcctgg cctttttgctg gccttttgc 4729
cacatgttct ttctgcgtt atccctgat tctgtggata accgtattac cgcctttgag 4789
tgagctgata ccgtcgcgcg cagccgaacg accgagcgcga gcgagtcagt gagcgaggaa 4849
gcggaagagc gcctgatgcg gtattttctc cttacgcata tgtgcggtat ttcacaccgc 4909
agaccagccg cgtaacctgg caaaatcggc tacggttgag taataaatgg atgccctgcg 4969
taagcgggtg tgggcggaca ataaagtctt aaactgaaca aaatagatct aaactatgac 5029
aataaagtct taaactagac agaatagttg taaactgaaa tcagtccagt tatgctgtga 5089
aaaagcatac tggacttttg ttatggctaa agcaaaactct tcattttctg aagtgcaaata 5149
tgcccgctgt attaaagagg ggcgtggcca agggcatggt aaagactata ttcgcggcgt 5209
tgtgacaatt taccgaacaa ctccgcggcc gggaagccga tctcggcttg aacgaattgt 5269
taggtggcgg tacttgggtc gatatacaag tgcatactt cttcccgat gcccaacttt 5329
gtatagagag cactgcggg atcgtcaccg taatctgctt gcacgtagat cacataagca 5389

ccaagcgcggt tggcctcatg cttgaggaga ttgatgagcg cgggtggcaat gccctgcctc 5449
cgggtgctcgc cggagactgc gagatcatag atatagatct cactacgcgg ctgctcaaac 5509
ctgggcagaa cgtaagccgc gagagcgcca 'acaaccgctt cttggtcgaa ggcagcaagc 5569
gcgatgaatg tcttactacg gagcaagttc ccgaggtaat cggagtccgg ctgatgttgg 5629
gagtaggtgg ctacgtctcc gaactcacga ccgaaaagat caagagcagc ccgcatggat 5689
ttgacttgggt cagggccgag cctacatgtg cgaatgatgc ccatacttga gccacctaac 5749
tttgtttttag ggcgactgcc ctgctgcgta acatcggtgc tgctgcgtaa catcggttgc 5809
gctccataac atcaaacatc gacccacggc gtaacgcgct tgctgcttgg atgcccgagg 5869
catagactgt acaaaaaaac agtcataaca agccatgaaa accgccactg cgccgttacc 5929
accgctgcgt tcgggtcaagg ttctggacca gttgcgtgag cgcatacgt acttgcat 5989
cagtttacga accgaacagg cttatgtcaa ctgggttcgt gccttcatcc gtttccacgg 6049
tgtgcgtcac ccggcaacct tgggcagcag cgaagtcgag gcatttctgt cctggctggc 6109
gaacgagcgc aagggttcgg tctccacgca tcgtcaggca ttggcggcct tgctgttctt 6169
ctacggcaag gtgctgtgca cggatctgcc ctggcttcag gagatcggtg gacctcggcc 6229
gtcgcggcgc ttgccggtgg tgctgacccc ggatgaagtg gttcgcaccc tcggttttct 6289
ggaaggcgag catcgtttgt tcgcccagga ctctagctat agttctagt gttggcctac 6349
gtacccgtag tggctatggc agggcttggc gccccgacgt tggctgcgag ccctgggcct 6409
tcacccgaac ttgggggttg ggggtgggaa aaggaagaaa cgcgggcgta ttggtcccaa 6469
tgggggtctcg gtgggggtatc gacagagtgc cagccctggg accgaacccc gcgtttatga 6529
acaaacgacc caacacccgt gcgttttatt ctgtcttttt attgccgtca tagcgcgggt 6589
tccttccgggt attgtctcct tccgtgtttc agttagcctc ccccatctcc cggtagcgca 6649
tgccctcgaga ctgcaggctc tagattcgaa agcggccgcg actagttagc tcgtcgacgt 6709
aggcctttga attccggatc ctcaactcaag gtcctcatca gagacgggtc tgatccagcg 6769
gcccagccga ccagggggtc tctgtgttgg agcattgggt tttggcttgg gctttggtag 6829
agcccgcctg ggattgcgat gcttcatctc catcgcagtc aagagcagat ctttcatctg 6889
ttcttggttt gggccacgtc catggttgat ttcatagact ttggcaactt cgtctatgaa 6949
agcttgggggt ggctctgcct gtcctggagc cccgtagatc gacgtagctg cccttaggat 7009
ttgttcttct gatgccaacc ggctcttctc tgcatgcacg tagtctagat agtcctcggt 7069
tgggtccgggt atttctcggt tgttctgcca gtactttacc tggcctgggc ttggccctcg 7129
gtgcccattg agtgctaccc attctgggtg tgcaaagtag atgcccattg tctccatctt 7189


```

ctttgagatc cgtgtgtctt tttccctctg tgcttcctct ggtgtggggc cccgagcctc 7249
cactccgtag cctgctgtcc cgtacttggc cctttgogac ttgctgcctg cttgtggtgc 7309
gtttgcaaga aaatttgcga tccgatgggc gttcgggtcg ctgagtgcga agttggccat 7369
gtcagtcaca atcccattct cttccagcca catgaacaca ctgagtgcag attggaatag 7429
tgggtccaag ttggtgtctg cttccattgc tctgacggca ctctcgagtt cgggggtctc 7489
tttgaactct gatgcagcca tggcgccctg aaaatacagg ttttcgggtcg ttgggatatc 7549
gtaatcgtga tgggtgatgg gatggtagta cgacatgggt tcggac 7595

```

<210> 4
 <211> 333
 <212> PRT
 <213> Artificial sequence

<220>
 <223> pVP2-his-VP3 protein

<400> 4
 Met Thr Asn Leu Ser Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg
 1 5 10 15
 Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
 20 25 30
 Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
 35 40 45
 Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
 50 55 60
 Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr
 65 70 75 80
 Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr
 85 90 95
 Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr
 100 105 110
 Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr
 115 120 125
 Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu
 130 135 140
 Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val
 145 150 155 160
 Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly
 165 170 175
 Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys
 180 185 190

Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile
 195 200 205

Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly
 210 215 220

Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu
 225 230 235 240

Ser Val Gly Gly Glu Leu Val Phe Arg Thr Ser Val His Gly Leu Val
 245 250 255

Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile
 260 265 270

Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn
 275 280 285

Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro
 290 295 300

Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln
 305 310 315 320

Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu
 325 330

<210> 5
 <211> 35
 <212> DNA
 <213> Artificial sequence

<220> Synthetic DNA
 <223> Oligo III primer

<400> 5
 gcgcagatct atgacaaacc tgtcagatca aaccc

35

<210> 6
 <211> 34
 <212> DNA
 <213> Artificial sequence

<220> Synthetic DNA
 <223> Oligo IV primer

<400> 6
 gcgcaagctt aggcgagagt cagctgcctt atgc

34

<210> 7
 <211> 9600
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Plasmid pESCURA/pVP2-VP3-GFP

<220>
 <221> promoter
 <222> (5649)..(5859)
 <223> Promoter 1 (pVP2)

<220>
 <221> promoter
 <222> (7402)..(8080)
 <223> Promoter 2 (VP3-GFP)

<220>
 <221> CDS
 <222> (8086)..(9597)
 <223> VP3-GFP ORF

<400> 7
 ggccgcacta gtatcgatgg attacaagga tgacgacgat aagatctgag ctcttaatta 60
 acaattcttc gccagaggtt tggccaagtc tccaatcaag gttgtcggct tgtctacctt 120
 gccagaaatt tacgaaaaga tggaaaaggg tcaaactcgtt ggtagatacg ttgttgacac 180
 ttctaaataa gcgaatttct tatgatttat gatttttatt attaaataag ttataaaaaa 240
 aataagtgtg tacaaatttt aaagtgactc ttaggtttta aaacgaaaat tcttattctt 300
 gagtaactct ttctgtagg tcaggttgct ttctcaggta tagcatgagg tcgctccaat 360
 tcagctgcat taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta ttgggcgctc 420
 ttccgcttcc tcgctcactg actcgctgcg ctcggtcggt cggtgcggc gagcggtatc 480
 agctcactca aaggcggtaa tacggttata cacagaatca ggggataacg caggaaagaa 540
 catgtgagca aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt tgcgtggcgtt 600
 ttcccatagg ctccgcccc ctgacgagca tcacaaaaat cgacgctcaa gtcagaggtg 660
 gcgaaacccg acaggactat aaagatacca ggcgtttccc cctggaagct ccctcgctgcg 720
 ctctcctggt ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc cttegggaag 780
 cgtggcgctt tctcatagct cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc 840
 caagctgggc tgtgtgcacg aacccccgt tcagcccgac cgctgcgcct tatccggtaa 900
 ctatcgtctt gagtccaacc cggtaaagaca cgacttatcg ccaactggcag cagccactgg 960
 taacaggatt agcagagcga ggtatgtagg cgggtgtaca gagttcttga agtggtggcc 1020
 taactacggc tacactagaa ggacagtatt tggatatctgc gctctgctga agccagttac 1080
 cttcggaaaa agagttggta gctcttgatc cggcaaacia accaccgctg gtagcgggtg 1140
 tttttttggt tgcaagcagc agattacgcg cagaaaaaaa ggatctcaag aagatccttt 1200
 gatcttttct acggggtctg acgctcagtg gaacgaaaac tcacgttaag ggattttggt 1260

catgagatta tcaaaaagga tcttcaccta gatcctttta aattaaat gaagtttta 1320
atcaatctaa agtatatatg agtaaacttg gtctgacagt taccaatgct taatcagtga 1380
ggcacctatc tcagcgatct gtctatttctg ttcattcata gttgcctgac tccccgtcgt 1440
gtagataact acgatacggg agggcttacc atctggcccc agtgctgcaa tgataccgcg 1500
agaccacgc tcaccggctc cagatttacc agcaataaac cagccagccg gaagggccga 1560
gcgcagaagt ggtcctgcaa ctttatccgc ctccatccag tctattaatt gttgccggga 1620
agctagagta agtagttcgc cagttaatag tttgcgcaac gttgttgcca ttgctacagg 1680
catcgtggtg tcacgctcgt cgtttggtat ggcttcattc agctccggtt cccaacgac 1740
aaggcgagtt acatgatccc ccatgttggtg caaaaaagcg gttagctcct tcggtcctcc 1800
gatcgttgctc agaagtaagt tggcgcagc gttatcactc atgggttatgg cagcactgca 1860
taattctctt actgtcatgc catccgtaag atgcttttct gtgactgggtg agtactcaac 1920
caagtcattc tgagaatagt gtatgcggcg accgagttgc tcttgcccg cgtcaatac 1980
ggataatacc gcgccacata gcagaacttt aaaagtgtc atcattggaa aacgttcttc 2040
ggggcgaaaa ctctcaagga tcttaccgct gttgagatcc agttcgatgt aaccactcg 2100
tgcacccaac tgatcttcag catcttttac tttcaccagc gtttctgggt gagcaaaaac 2160
aggaaggcaa aatgccgcaa aaaaggaat aaggcgaca cggaaatgtt gaatactcat 2220
actcttcctt tttcaatatt attgaagcat ttatcagggt tattgtctca tgagcggata 2280
catatttgaa tgtatttaga aaaataaaca aataggggtt ccgcgacat tccccgaaa 2340
agtgccaact gaacgaagca tctgtgcttc atttttaga acaaaaatgc aacgcgagag 2400
cgctaatttt tcaacaaag aatctgagct gcatttttac agaacagaaa tgcaacgcga 2460
aagcgctatt ttaccaacga agaattctgtg cttcattttt gtaaaacaaa aatgcaacgc 2520
gagagcgcta atttttcaaa caaagaatct gagctgcatt ttacagaa agaaatgcaa 2580
cgcgagagcg ctattttacc aacaaagaat ctatacttct ttttgttct acaaaaatgc 2640
atcccgagag cgctattttt ctaacaaagc atcttagatt acttttttcc tctttgtgc 2700
gctctataat gcagtcctt gataactttt tgcaactgtg gtccgttaag gtagaagaa 2760
ggctactttg gtgtctattt tctcttccat aaaaaagcc tgactccact tccgcgttt 2820
actgattact agcgaagctg cgggtgcatt ttttcaagat aaaggcatcc ccgattatat 2880
tctataccga tgtggattgc gcatactttg tgaacagaaa gtgatagcgt tgatgattct 2940
tcattggtca gaaaattatg aacggtttct tctattttgt ctctatatac tacgtatagg 3000
aatggtttac attttctgtat tgttttcgat tcaactctatg aatagttctt actacaattt 3060

ttttgtctaa agagtaatac tagagataaa cataaaaaat gtagaggctcg agtttagatg 3120
caagttcaag gagcgaaagg tggatgggta gggtatatag ggatatagca cagagatata 3180
tagcaaagag atacttttga gcaatgtttg tggaagcggg attcgcaata ttttagtagc 3240
tcgttacagt ccggtgcgtt tttggttttt tgaaagtgcg tcttcagagc gcttttggtt 3300
ttcaaaagcg ctctgaagtt cctatacttt ctagagaata ggaacttcgg aataggaact 3360
tcaaagcgtt tccgaaaacg agcgcttcgg aaaatgcaac gcgagctgcg cacatacagc 3420
tcaactgttca cgtcgcacct atatctgcgt gttgcctgta tatatatata catgagaaga 3480
acggcatagt gcgtgtttat gcttaaattgc gtacttatat gcgtctatct atgtaggatg 3540
aaaggtagtc tagtacctcc tgtgatatta tcccattcca tgcgggggat cgtatgcttc 3600
cttcagcact acccttttagc tggtctatat gctgccactc ctcaattgga ttagtctcat 3660
ccttcaatgc tatcatttcc tttgatattg gatcatacta agaaaccatt attatcatga 3720
cattaaccta taaaaatagg cgtatcacga ggccctttcg tctcgcgcggt ttcgggtgatg 3780
acggtgaaaa cctctgacac atgcagctcc cggagacggg cacagcttgt ctgtaagcgg 3840
atgccgggag cagacaagcc cgtcagggcg cgtcagcggg tggtggcggg tgcgggggct 3900
ggcttaacta tgcggcatca gagcagattg tactgagagt gcaccatacc acagcttttc 3960
aattcaattc atcatttttt ttttattctt ttttttgatt tcggtttctt tgaaattttt 4020
ttgattcggg aatctccgaa cagaaggaag aacgaaggaa ggagcacaga cttagattgg 4080
tatatatacg catatgtagt gttgaagaaa catgaaattg ccagtatctc ttaaccaaac 4140
tgcacagaac aaaaacctgc aggaacgaa gataaatcat gtcgaaagct acatataagg 4200
aacgtgctgc tactcatcct agtcctgttg ctgccaagct atttaatatc atgcacgaaa 4260
agcaaacaaa cttgtgtgct tcattggatg ttcgtaccac caaggaatta ctggagttag 4320
ttgaagcatt aggtcccaaa atttgtttac taaaaacaca tgtggatata ttgactgatt 4380
tttccatgga gggcacagtt aagccgctaa aggcattatc cgccaagtac aattttttac 4440
tcttcgaaga cagaaaattt gctgacattg gtaatacagt caaattgcag tactctgcgg 4500
gtgtatacag aatagcagaa tgggcagaca ttacgaatgc acacgggtgtg gtgggcccag 4560
gtattgttag cggtttgaag caggcggcag aagaagtaac aaaggaacct agaggccttt 4620
tgatgttagc agaattgtca tgcaagggct ccctatctac tggagaatat actaagggtg 4680
ctgttgacat tgogaagagc gacaaagatt ttgttatcgg ctttattgct caaagagaca 4740
tgggtggaag agatgaagggt tacgattggg tgattatgac acccggtgtg ggtttagatg 4800
acaagggaga cgcattgggt caacagtata gaaccgtgga tgatgtggct tctacaggat 4860

ctgacattat tattgttggga agaggactat ttgcaaaggg aagggatgct aaggtagagg 4920
gtgaacgtta cagaaaagca ggctgggaag catatttgag aagatgcggc cagcaaaact 4980
aaaaaactgt attataagta aatgcatgta tactaaactc acaaattaga gcttcaattt 5040
aattatatca gttattaccc tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa 5100
taccgcatca ggaaattgta aacgttaata ttttgttaaa attcgcgtta aatttttgtt 5160
aatcagctc attttttaac caataggccg aaatcgga aatcccttat aaatcaaaag 5220
aatagaccga gatagggttg agtggtgttc cagtttggaa caagagtcca ctattaaaga 5280
acgtggactc caacgtcaaa gggcgaaaaa cgtctatca gggcgatggc ccactacgtg 5340
aaccatcacc ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc 5400
ctaaagggag ccccgattt agagcttgac ggggaaagcc gggaacgtg gcgagaaagg 5460
aagggaagaa agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc 5520
gcgtaaccac cacaccgcc gcgcttaatg cgccgctaca gggcgcgctc cgccattcgc 5580
cattcaggct gcgcaactgt tgggaagggc gatcggtgcg ggccctcttcg ctattacgcc 5640
agctggatct tcgagcgtcc caaaaccttc tcaagcaagg ttttcagtat aatgttacat 5700
gcgtacacgc gtctgtacag aaaaaaaga aaaatttgaa atataaataa cgttcttaat 5760
actaacataa ctataaaaaa ataaataggg acctagactt caggttgtct aactccttcc 5820
ttttcggtta gagcggatct tagctagccg cggtagcaag cttaggcgag agtcagctgc 5880
cttatgcggc ctgaggcagc tcttgctttt cctgacgcgg ctogagcagt tctgaagcg 5940
gcctgggct catcgcccag caggtagtct acacctccc caattgcatg ggctagggga 6000
gcggcaggtg ggaacaatgt ggagaccacc ggcacagcta tctccttat ggcccgatt 6060
atgtctttga agccgaatgc tctgcaatc ttcaggggag agttgaggtc ggccacctcc 6120
atgaagtatt cacgaaagtc agtgactcc cttgttggcc agacggtctt gatgccaaga 6180
cggctccctc cactcagtat caattttggt tagttcatgg ctctgggtc aaatcggccg 6240
tattctgtaa ccaggttctt tgctagttca ggatttggga tcagctcgaa gttgctcacc 6300
ccagcgaccg taacgacgga tctgttgcc actctttcgt aggccactag cgtgacggga 6360
cggagggccc ctggatagtt gccaccatgg atcgactg ctaggctccc tcttgccgac 6420
catgacatct gatcccctgc ctgaccacca cttttggagg tcaactatctc cagtttgatg 6480
gatgtgattg gctgggttat ctggttggtt ggaatcacia gattgaatgg cataagggtg 6540
tcggtgcgg tcgtcagccc attgtttgcg gccacagccc tgggtgattac cgttggtcca 6600
tcaaagccta tgaggtagat ggtggcgccc agtacaaggc cgtggacgct tgttcgaaac 6660

| | | | | | | | |
|-------------|-------------|-------------|-------------|-------------|-----------------|--|------|
| acgagctctc | ccccaacgct | gaggcttggtg | atggcatcaa | tgttggctga | gaacagtgtg | | 6720 |
| attgttacct | cacctgggtg | gtactgtgat | gagaattggg | aatcatcggc | tgcagttatg | | 6780 |
| gtgtagactc | tgggcctgtc | actgctgtca | catgtggcta | ccatttttgg | gtcaagccct | | 6840 |
| attgcgggaa | tggggtcacc | aagcctcaca | taccaagat | catatgatgt | gggtaagctg | | 6900 |
| aggacggtga | ccccttcccc | tactaggacg | ttcccaattt | tgtcgttgat | gttggctggt | | 6960 |
| gcagacatca | accatttgta | gctaacatct | gtcagttcac | tcaggcttcc | ttggaaggtc | | 7020 |
| acggcgttta | tgggtgccgtt | tagtgcataa | acgccaccag | gaagtgtgct | tgacctcaact | | 7080 |
| gtgagactcc | gactcactag | cctgcagtag | ttgtaactgg | ccggtagggt | ctgggcagtc | | 7140 |
| aggagcatct | gatcgaactt | gtagttccca | ttgccctgca | gtgtgtagtg | agcaccacaca | | 7200 |
| attgagccag | ggaatccagg | gaaaaagaca | attagccctg | accctgtgtc | ccccacagtc | | 7260 |
| aaattgtagg | tcgaggtctc | tgacctgaga | gtgtgcttct | ccagggtgtc | gtccggaatg | | 7320 |
| gacgccggtc | cggttggttg | catcagaagg | ctccgtatga | acggaacaat | ctgctggggt | | 7380 |
| tgatctgaca | ggtttgatcat | agatccgggg | ttttttctcc | ttgacgttaa | agtatagagg | | 7440 |
| tatattaaca | atTTTTTgtt | gatactttta | ttacatttga | ataagaagta | atacaaaccg | | 7500 |
| aaaatgttga | aagtattagt | taaagtgggt | atgcagtttt | tgcatttata | tatctgttaa | | 7560 |
| tagatcaaaa | atcatcgctt | cgctgattaa | ttaccccaga | aataaggcta | aaaaactaat | | 7620 |
| cgcattatca | tcctatgggt | gttaatttga | ttcgttcatt | tgaaggtttg | tggggccagg | | 7680 |
| ttactgccaa | tttttctctc | tcataaccat | aaaagctagt | attgtagaat | ctttattggt | | 7740 |
| cggagcagtg | cggcgcgagg | cacatctgcg | tttcaggaac | gcgaccgggtg | aagacgagga | | 7800 |
| cgcacggagg | agagtcttcc | ttcggagggc | tgtcaccgcg | tcggcggctt | ctaataccgta | | 7860 |
| cttcaatata | gcaatgagca | gttaagcgta | ttactgaaag | ttccaaagag | aaggtttttt | | 7920 |
| taggctaaga | taatggggct | ctttacattt | ccacaacata | taagtaagat | tagatatgga | | 7980 |
| tatgtatatg | gatatgtata | tgggtggaat | gccatgtaat | atgattatta | aacttctttg | | 8040 |
| cgtccatcca | aaaaaaaaagt | aagaattttt | gaaaattcga | attcg | atg gct gca tca | | 8097 |
| | | | | | Met Ala Ala Ser | | |
| | | | | | 1 | | |
| gag ttc aaa | gag acc ccc | gaa ctc | gag agt gcc | gtc aga gca | atg gaa | | 8145 |
| Glu Phe Lys | Glu Thr Pro | Glu Leu | Glu Ser Ala | Val Arg Ala | Met Glu | | |
| 5 | 10 | | 15 | | 20 | | |
| gca gca gcc | aac gtg gac | cca cta ttc | caa tct gca | ctc agt gtg | ttc | | 8193 |
| Ala Ala Ala | Asn Val Asp | Pro Leu Phe | Gln Ser Ala | Leu Ser Val | Phe | | |
| | 25 | | 30 | | 35 | | |
| atg tgg ctg | gaa gag aat | ggg att gtg | act gac atg | gcc aac ttc | gca | | 8241 |

| | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| Met | Trp | Leu | Glu | Glu | Asn | Gly | Ile | Val | Thr | Asp | Met | Ala | Asn | Phe | Ala | |
| | | 40 | | | | | | 45 | | | | | 50 | | | |
| ctc | agc | gac | ccg | aac | gcc | cat | cgg | atg | cga | aat | ttt | ctt | gca | aac | gca | 8289 |
| Leu | Ser | Asp | Pro | Asn | Ala | His | Arg | Met | Arg | Asn | Phe | Leu | Ala | Asn | Ala | |
| | | 55 | | | | | 60 | | | | | 65 | | | | |
| cca | caa | gca | ggc | agc | aag | tcg | caa | agg | gcc | aag | tac | ggg | aca | gca | ggc | 8337 |
| Pro | Gln | Ala | Gly | Ser | Lys | Ser | Gln | Arg | Ala | Lys | Tyr | Gly | Thr | Ala | Gly | |
| | 70 | | | | | 75 | | | | | 80 | | | | | |
| tac | gga | gtg | gag | gct | cgg | ggc | ccc | aca | cca | gag | gaa | gca | cag | agg | gaa | 8385 |
| Tyr | Gly | Val | Glu | Ala | Arg | Gly | Pro | Thr | Pro | Glu | Glu | Ala | Gln | Arg | Glu | |
| 85 | | | | | 90 | | | | | 95 | | | | | 100 | |
| aaa | gac | aca | cgg | atc | tca | aag | aag | atg | gag | acc | atg | ggc | atc | tac | ttt | 8433 |
| Lys | Asp | Thr | Arg | Ile | Ser | Lys | Lys | Met | Glu | Thr | Met | Gly | Ile | Tyr | Phe | |
| | | | | 105 | | | | | 110 | | | | | 115 | | |
| gca | aca | cca | gaa | tgg | gta | gca | ctc | aat | ggg | cac | cga | ggg | cca | agc | cca | 8481 |
| Ala | Thr | Pro | Glu | Trp | Val | Ala | Leu | Asn | Gly | His | Arg | Gly | Pro | Ser | Pro | |
| | | | 120 | | | | | 125 | | | | | 130 | | | |
| ggc | cag | gta | aag | tac | tgg | cag | aac | aaa | cga | gaa | ata | ccg | gac | cca | aac | 8529 |
| Gly | Gln | Val | Lys | Tyr | Trp | Gln | Asn | Lys | Arg | Glu | Ile | Pro | Asp | Pro | Asn | |
| | | 135 | | | | | 140 | | | | | 145 | | | | |
| gag | gac | tat | cta | gac | tac | gtg | cat | gca | gag | aag | agc | cgg | ttg | gca | tca | 8577 |
| Glu | Asp | Tyr | Leu | Asp | Tyr | Val | His | Ala | Glu | Lys | Ser | Arg | Leu | Ala | Ser | |
| | 150 | | | | | 155 | | | | | 160 | | | | | |
| gaa | gaa | caa | atc | cta | agg | gca | gct | acg | tcg | atc | tac | ggg | gct | cca | gga | 8625 |
| Glu | Glu | Gln | Ile | Leu | Arg | Ala | Ala | Thr | Ser | Ile | Tyr | Gly | Ala | Pro | Gly | |
| 165 | | | | | 170 | | | | | 175 | | | | 180 | | |
| cag | gca | gag | cca | ccc | caa | gct | ttc | ata | gac | gaa | gtt | gcc | aaa | gtc | tat | 8673 |
| Gln | Ala | Glu | Pro | Pro | Gln | Ala | Phe | Ile | Asp | Glu | Val | Ala | Lys | Val | Tyr | |
| | | | | 185 | | | | | 190 | | | | | 195 | | |
| gaa | atc | aac | cat | gga | cgt | ggc | cca | aac | caa | gaa | cag | atg | aaa | gat | ctg | 8721 |
| Glu | Ile | Asn | His | Gly | Arg | Gly | Pro | Asn | Gln | Glu | Gln | Met | Lys | Asp | Leu | |
| | | 200 | | | | | | 205 | | | | | 210 | | | |
| ctc | ttg | act | gcg | atg | gag | atg | aag | cat | cgc | aat | ccc | agg | cgg | gct | cta | 8769 |
| Leu | Leu | Thr | Ala | Met | Glu | Met | Lys | His | Arg | Asn | Pro | Arg | Arg | Ala | Leu | |
| | | 215 | | | | | 220 | | | | | 225 | | | | |
| cca | aag | ccc | aag | cca | aaa | ccc | aat | gct | cca | aca | cag | aga | ccc | cct | ggt | 8817 |
| Pro | Lys | Pro | Lys | Pro | Lys | Pro | Asn | Ala | Pro | Thr | Gln | Arg | Pro | Pro | Gly | |
| | 230 | | | | | 235 | | | | | 240 | | | | | |
| cgg | ctg | ggc | cgc | tgg | atc | agg | acc | gtc | tct | gat | gag | gac | ctt | gag | gga | 8865 |
| Arg | Leu | Gly | Arg | Trp | Ile | Arg | Thr | Val | Ser | Asp | Glu | Asp | Leu | Glu | Gly | |
| 245 | | | | | 250 | | | | | 255 | | | | 260 | | |
| toc | atc | gcc | acc | atg | gtg | agc | aag | ggc | gag | gag | ctg | ttc | acc | ggg | gtg | 8913 |
| Ser | Ile | Ala | Thr | Met | Val | Ser | Lys | Gly | Glu | Glu | Leu | Phe | Thr | Gly | Val | |
| | | | | 265 | | | | | 270 | | | | | 275 | | |
| gtg | ccc | atc | ctg | gtc | gag | ctg | gac | ggc | gac | gta | aac | ggc | cac | aag | ttc | 8961 |

<210> 8
 <211> 503
 <212> PRT
 <213> Artificial sequence

<220>
 <223> pVP2-VP3-GFP protein

<400> 8
 Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala Val
 1 5 10 15
 Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser Ala
 20 25 30
 Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp Met
 35 40 45
 Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn Phe
 50 55 60
 Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys Tyr
 65 70 75 80
 Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu Glu
 85 90 95
 Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr Met
 100 105 110
 Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His Arg
 115 120 125
 Gly Pro Ser Pro Gly Gln Val Lys Tyr Trp Gln Asn Lys Arg Glu Ile
 130 135 140
 Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys Ser
 145 150 155 160
 Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile Tyr
 165 170 175
 Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu Val
 180 185 190
 Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu Gln
 195 200 205
 Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn Pro
 210 215 220
 Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn Ala Pro Thr Gln
 225 230 235 240
 Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr Val Ser Asp Glu
 245 250 255
 Asp Leu Glu Gly Ser Ile Ala Thr Met Val Ser Lys Gly Glu Glu Leu
 260 265 270

Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn
 275 280 285
 Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr
 290 295 300
 Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val
 305 310 315 320
 Pro Trp Pro Thr Leu Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe
 325 330 335
 Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala
 340 345 350
 Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp
 355 360 365
 Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu
 370 375 380
 Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn
 385 390 395 400
 Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr
 405 410 415
 Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile
 420 425 430
 Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln
 435 440 445
 Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His
 450 455 460
 Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg
 465 470 475 480
 Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu
 485 490 495
 Gly Met Asp Glu Leu Tyr Lys
 500

<210> 9
 <211> 33
 <212> DNA
 <213> Artificial sequence

<220> Synthetic DNA
 <223> Oligo V primer

<400> 9
 gcgcgaattc gatggcatca gagttcaaag aga

<210> 10
 <211> 32

<212> DNA
<213> Artificial sequence

<220> Synthetic DNA
<223> Oligo VI primer

<400> 10
cgcggtatccc tcaaggtcct catcagagac gg 32

<210> 11
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Oligo CD8 A primer

<400> 11
aacgaggaca gttatgtccc aagcgcagaa caaata 36

<210> 12
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Oligo CD8 B primer

<400> 12
tattttgttct gcgcttgga cataactgtc ctcggtt 36

<210> 13
<211> 5676
<212> DNA
<213> Artificial sequence

<220>
<223> Plasmid pFB/his-CD8-VP3

<220>
<221> promoter
<222> (1)..(129)
<223> Polyhedrin promoter

<220>
<221> CDS
<222> (147)..(1043)
<223> His-CD8-VP3 ORF

<220>
<221> CDS
<222> (222)..(257)
<223> His-CD8 ORF

<400> 13
atcatggaga taattaaaat gataaccatc tcgcaaataa ataagtattt tactgttttc 60
gtaacagttt tgtaataaaa aaacctataa atattccgga ttattcatat cgtcccacca 120

| | |
|---|-----|
| tctgggcgcggg atctcgggtcc gaaacc atg tcg tac tac cat cac cat cac cat | 173 |
| Met Ser Tyr Tyr His His His His His | |
| 1 5 | |
| cac gat tac gat atc cca acg acc gaa aac ctg tat ttt cag ggc gcg | 221 |
| His Asp Tyr Asp Ile Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala | |
| 10 15 20 25 | |
| aac gag gac agt tat gtc cca agc gca gaa caa ata gcc gcc atg gct | 269 |
| Asn Glu Asp Ser Tyr Val Pro Ser Ala Glu Gln Ile Ala Ala Met Ala | |
| 30 35 40 | |
| gca tca gag ttc aaa gag acc ccc gaa ctc gag agt gcc gtc aga gca | 317 |
| Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala Val Arg Ala | |
| 45 50 55 | |
| atg gaa gca gca gcc aac gtg gac cca cta ttc caa tct gca ctc agt | 365 |
| Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser Ala Leu Ser | |
| 60 65 70 | |
| gtg ttc atg tgg ctg gaa gag aat ggg att gtg act gac atg gcc aac | 413 |
| Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp Met Ala Asn | |
| 75 80 85 | |
| ttc gca ctc agc gac ccg aac gcc cat cgg atg cga aat ttt ctt gca | 461 |
| Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn Phe Leu Ala | |
| 90 95 100 105 | |
| aac gca cca caa gca ggc agc aag tcg caa agg gcc aag tac ggg aca | 509 |
| Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys Tyr Gly Thr | |
| 110 115 120 | |
| gca ggc tac gga gtg gag gct cgg ggc ccc aca cca gag gaa gca cag | 557 |
| Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu Glu Ala Gln | |
| 125 130 135 | |
| agg gaa aaa gac aca cgg atc tca aag aag atg gag acc atg ggc atc | 605 |
| Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr Met Gly Ile | |
| 140 145 150 | |
| tac ttt gca aca cca gaa tgg gta gca ctc aat ggg cac cga ggg cca | 653 |
| Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His Arg Gly Pro | |
| 155 160 165 | |
| agc cca ggc cag gta aag tac tgg cag aac aaa cga gaa ata ccg gac | 701 |
| Ser Pro Gly Gln Val Lys Tyr Trp Gln Asn Lys Arg Glu Ile Pro Asp | |
| 170 175 180 185 | |
| cca aac gag gac tat cta gac tac gtg cat gca gag aag agc cgg ttg | 749 |
| Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys Ser Arg Leu | |
| 190 195 200 | |
| gca tca gaa gaa caa atc cta agg gca gct acg tcg atc tac ggg gct | 797 |
| Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile Tyr Gly Ala | |
| 205 210 215 | |

| | |
|---|------|
| cca gga cag gca gag cca ccc caa gct ttc ata gac gaa gtt gcc aaa Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu Val Ala Lys 220 225 230 | 845 |
| gtc tat gaa atc aac cat gga cgt ggc cca aac caa gaa cag atg aaa Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu Gln Met Lys 235 240 245 | 893 |
| gat ctg ctc ttg act gcg atg gag atg aag cat cgc aat ccc agg cgg Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn Pro Arg Arg 250 255 260 265 | 941 |
| gct cta cca aag ccc aag cca aaa ccc aat gct cca aca cag aga ccc Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn Ala Pro Thr Gln Arg Pro 270 275 280 | 989 |
| cct ggt cgg ctg ggc cgc tgg atc agg acc gtc tct gat gag gac ctt Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr Val Ser Asp Glu Asp Leu 285 290 295 | 1037 |
| gag tga ggatccggaa ttcaaaggcc tacgtcgacg agctcactag tcgcggccgc Glu | 1093 |
| tttcgaatct agagcctgca gtctcgaggc atgcggtacc aagcttgctg agaagtacta | 1153 |
| gaggatcata atcagccata ccacatttgt agaggtttta cttgctttaa aaaacctccc | 1213 |
| acacctcccc ctgaacctga aacataaaat gaatgcaatt gttgttggtta acttgtttat | 1273 |
| tgcagcttat aatggttaca aataaagcaa tagcatcaca aatttcacaa ataaagcatt | 1333 |
| tttttactg cattctagtt gtggtttgtc caaactcatc aatgtatctt atcatgtctg | 1393 |
| gatctgatca ctagatctgc ctaggagatc cgaaccagat aagtgaaatc tagttccaaa | 1453 |
| ctattttgtc atttttaatt ttcgtattag cttacgacgc tacacccagt tcccatctat | 1513 |
| tttgtcactc ttccctaaat aatccttaaa aactccattt ccacctctcc cagttcccaa | 1573 |
| ctattttgtc cgccacagc ggggcatttt tcttctgtt atgtttttaa tcaaacatcc | 1633 |
| tgccaactcc atgtgacaaa cgcctatctt cggctacttt ttctctgtca cagaatgaaa | 1693 |
| atttttctgt catctcttcg ttattaatgt ttgtaattga ctgaatatca acgcttattt | 1753 |
| gcagcctgaa tggcgaatgg gacgcgccct gtagcggcgc attaagcgcg gcgggtgtgg | 1813 |
| tggttacgcg cagcgtgacc gctacacttg ccagcgcctt agcgcgcgt cctttcgctt | 1873 |
| tcttcccttc ctttctcgcc acgttcgccg gctttccccg tcaagctcta aatcgggggc | 1933 |
| tcccttttagg gttccgattt agtgctttac ggcacctga ccccaaaaaa cttgattagg | 1993 |
| gtgatggttc acgtagtggg ccatcgccct gatagacggt ttttcgccct ttgacgttgg | 2053 |
| agtccacggt ctttaatatgt ggaactcttg tccaaactgg aacaacactc aacctatct | 2113 |
| cggctatctc ttttgattta taagggtatt tgccgatttc ggcctattgg ttaaaaaatg | 2173 |

agctgattta acaaaaattt aacgcgaatt ttaacaaaat attaacgttt acaatttcag 2233
gtggcacttt tcggggaaat gtgcgcggaa cccctatttg tttatttttc taaatacatt 2293
caaatatgta tccgctcatg agacaataac cctgataaat gcttcaataa tattgaaaaa 2353
ggaagagtat gagtattcaa catttccgtg tcgcccttat tccctttttt gcggcatttt 2413
gccttcctgt ttttgctcac ccagaaacgc tggtgaaagt aaaagatgct gaagatcagt 2473
tggtgtcacg agtgggttac atcgaaactgg atctcaacag cggtaagatc cttgagagtt 2533
ttcgccccga agaacgtttt ccaatgatga gcacttttaa agttctgcta tgtggcgcg 2593
tattatcccg tattgacgcc gggcaagagc aactcggctg ccgcatacac tattctcaga 2653
atgacttggg tgagtactca ccagtcacag aaaagcatct tacggatggc atgacagtaa 2713
gagaattatg cagtgtgcc ataaccatga gtgataaac tgccggccaac ttacttctga 2773
caacgatcgg aggaccgaag gagctaaccg cttttttgca caacatgggg gatcatgtaa 2833
ctcgccttga tcgttgggaa ccggagctga atgaagccat accaaacgac gagcgtgaca 2893
ccacgatgcc tgtagcaatg gcaacaacgt tgcgcaaact attaaactggc gaactactta 2953
ctctagcttc ccggcaacaa ttaatagact ggatggaggc ggataaagtt gcaggaccac 3013
ttctgcgctc ggcccttcg gctggctggg ttattgctga taaatctgga gccggtgagc 3073
gtgggtctcg cggatatcatt gcagcactgg gccagatgg taagccctcc cgtatcgtag 3133
ttatctacac gacggggagt caggcaacta tggatgaacg aaatagacag atcgtgaga 3193
taggtgcctc actgattaag cattggtaac tgtcagacca agtttactca tatatacttt 3253
agattgattt aaaacttcat ttttaattta aaaggatcta ggtgaagatc ctttttgata 3313
atctcatgac caaaatccct taacgtgagt tttcgttcca ctgagcgtca gaccccgtag 3373
aaaagatcaa aggatcttct tgagatcctt tttttctgcg cgtaatctgc tgcttgcaaa 3433
caaaaaaacc accgctaoca gcggtggttt gtttgccgga tcaagagcta ccaactcttt 3493
ttccgaagg t aactggcttc agcagagcgc agataccaaa tactgtcctt ctagtgtagc 3553
cgtagttagg ccaccacttc aagaactctg tagcaccgcc tacatacctc gctctgctaa 3613
tcctgttacc agtggctgct gccagtggcg ataagtcgtg tcttaccggg ttggactcaa 3673
gacgatagtt accggataag gcgcagcggc cgggctgaac ggggggttcg tgcacacagc 3733
ccagcttgga gcgaacgacc tacaccgaac tgagatacct acagcgtgag cattgagaaa 3793
gcgccacgct tcccgaagg agaaaaggcg acaggatatcc ggtaagcggc agggtcggaa 3853
caggagagcg cacgaggag cttccagggg gaaacgcctg gtatctttat agtcctgtcg 3913
ggtttcgcca cctctgactt gagcgtcgat ttttgtgatg ctgcgtcagg gggcggagcc 3973

tatggaaaaa cgccagcaac gcggcctttt tacggttcct ggcccttttg tggccttttg 4033
ctcacatgtt ctttcctgcg ttatccoctg attctgtgga taaccgtatt accgcctttg 4093
agttagctga taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca gtgagcgagg 4153
aagcggaaga gcgcctgatg cggatattttc tccttacgca tctgtgcggt atttcacacc 4213
gcagaccagc cgcgtaacct ggcaaaatcg gttacggttg agtaataaat ggatgccctg 4273
cgtaagcggg tgtgggcgga caataaagtc ttaaactgaa caaaatagat ctaaactatg 4333
acaataaagt cttaaactag acagaatagt tgtaaactga aatcagtcca gttatgctgt 4393
gaaaaagcat actggacttt tggttatggc aaagcaaact cttcattttc tgaagtgcaa 4453
attgcccgtc gtattaaaga ggggcgtggc caagggcatg gtaaagacta tattcgcggc 4513
gttgtgacaa ttaccgaac aactccgcg cggggaagcc gatctcggct tgaacgaatt 4573
gttaggtggc ggtacttggg tcgatatcaa agtgcacac ttcttcccgt atgcccaact 4633
ttgtatagag agccactgcg ggatcgtcac cgtaatctgc ttgcacgtag atcacataag 4693
caccaagcgc gttggcctca tgcttgagga gattgatgag cgcggtggca atgccctgcc 4753
tccggtgctc gccggagact gcgagatcat agatatagat ctactacgc ggctgctcaa 4813
acctgggcag aacgtaagcc gcgagagcgc caacaaccgc ttcttggtcg aaggcagcaa 4873
gcgcgatgaa tgtcttacta cggagcaagt tcccagagta atcggagtcc ggctgatgtt 4933
gggagtaggt ggctacgtct ccgaactcac gaccgaaaag atcaagagca gcccgcatgg 4993
atttgacttg gtcagggcgc agcctacatg tgcgaaatgat gcccatactt gagccaccta 5053
actttgtttt agggcgactg ccctgctgcg taacatcggt gctgctgctg aacatcggtg 5113
ctgctccata acatcaaaca tcgaccacgc gcgtaacgcg cttgctgctt ggatgccga 5173
ggcatagact gtacaaaaaa acagtcataa caagccatga aaaccgccac tgcgcgtta 5233
ccaccgctgc gttcgggtcaa ggttctggac cagttgcgtg agcgcatacg ctacttgcac 5293
tacagtttac gaaccgaaca ggcttatgtc aactgggttc gtgccttcat ccgtttccac 5353
ggtgtgcgtc acccggaac cttgggcagc agcgaagtcg aggcatttct gtccctggctg 5413
gcgaacgagc gcaagggttc ggtctccacg catcgtcagg cattggcggc cttgctgttc 5473
ttctacggca aggtgctgtg cacggatctg ccctggcttc aggagatcgg aagacctcg 5533
ccgtcgcggc gcttgccggt ggtgctgacc ccggatgaag tggttcgcac cctcggtttt 5593
ctggaaggcg agcatcggtt gttcgcccag gactctagct atagttctag tggttggcta 5653
cgtatactcc ggaatattaa tag 5676

<210> 14
 <211> 298
 <212> PRT
 <213> Artificial sequence

<220>
 <223> his-CD8-VP3 protein

<400> 14

| | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Tyr | Tyr | His | His | His | His | His | His | Asp | Tyr | Asp | Ile | Pro | Thr | 1 | 5 | 10 | 15 |
| Thr | Glu | Asn | Leu | Tyr | Phe | Gln | Gly | Ala | Asn | Glu | Asp | Ser | Tyr | Val | Pro | 20 | 25 | 30 | |
| Ser | Ala | Glu | Gln | Ile | Ala | Ala | Met | Ala | Ala | Ser | Glu | Phe | Lys | Glu | Thr | 35 | 40 | 45 | |
| Pro | Glu | Leu | Glu | Ser | Ala | Val | Arg | Ala | Met | Glu | Ala | Ala | Ala | Asn | Val | 50 | 55 | 60 | |
| Asp | Pro | Leu | Phe | Gln | Ser | Ala | Leu | Ser | Val | Phe | Met | Trp | Leu | Glu | Glu | 65 | 70 | 75 | 80 |
| Asn | Gly | Ile | Val | Thr | Asp | Met | Ala | Asn | Phe | Ala | Leu | Ser | Asp | Pro | Asn | 85 | 90 | 95 | |
| Ala | His | Arg | Met | Arg | Asn | Phe | Leu | Ala | Asn | Ala | Pro | Gln | Ala | Gly | Ser | 100 | 105 | 110 | |
| Lys | Ser | Gln | Arg | Ala | Lys | Tyr | Gly | Thr | Ala | Gly | Tyr | Gly | Val | Glu | Ala | 115 | 120 | 125 | |
| Arg | Gly | Pro | Thr | Pro | Glu | Glu | Ala | Gln | Arg | Glu | Lys | Asp | Thr | Arg | Ile | 130 | 135 | 140 | |
| Ser | Lys | Lys | Met | Glu | Thr | Met | Gly | Ile | Tyr | Phe | Ala | Thr | Pro | Glu | Trp | 145 | 150 | 155 | 160 |
| Val | Ala | Leu | Asn | Gly | His | Arg | Gly | Pro | Ser | Pro | Gly | Gln | Val | Lys | Tyr | 165 | 170 | 175 | |
| Trp | Gln | Asn | Lys | Arg | Glu | Ile | Pro | Asp | Pro | Asn | Glu | Asp | Tyr | Leu | Asp | 180 | 185 | 190 | |
| Tyr | Val | His | Ala | Glu | Lys | Ser | Arg | Leu | Ala | Ser | Glu | Glu | Gln | Ile | Leu | 195 | 200 | 205 | |
| Arg | Ala | Ala | Thr | Ser | Ile | Tyr | Gly | Ala | Pro | Gly | Gln | Ala | Glu | Pro | Pro | 210 | 215 | 220 | |
| Gln | Ala | Phe | Ile | Asp | Glu | Val | Ala | Lys | Val | Tyr | Glu | Ile | Asn | His | Gly | 225 | 230 | 235 | 240 |
| Arg | Gly | Pro | Asn | Gln | Glu | Gln | Met | Lys | Asp | Leu | Leu | Leu | Thr | Ala | Met | 245 | 250 | 255 | |

Glu Met Lys His Arg Asn Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro
260 265 270

Lys Pro Asn Ala Pro Thr Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp
275 280 285

Ile Arg Thr Val Ser Asp Glu Asp Leu Glu
290 295

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N7/04 A61K39/12 C12N15/62 C07K14/08 C07K19/00
C12N15/87 C12N15/86 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|----------------------------|
| X | CHEVALIER C ET AL: "The maturation process of pVP2 requires assembly of infectious bursal disease virus capsids" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 76, no. 5, March 2002 (2002-03), pages 2384-2392, XP002218366 ISSN: 0022-538X the whole document | 1-4, 6, 9-14, 17-20, 23-29 |
| X | WO 02/088339 A (INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE ; DELMAS, BERNARD; CHEVA) 7 November 2002 (2002-11-07) page 2 - page 3 page 9 - page 10; claims 1-14 -/-- | 1-4, 6, 9-14, 17-20, 23-29 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

15 June 2005

Date of mailing of the international search report

11/07/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Paresce, D

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|--|-----------------------|
| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y | <p>HU Y ET AL: "Chimeric infectious bursal disease virus-like particles expressed in insect cells and purified by immobilized metal affinity chromatography"</p> <p>BIOTECHNOLOGY AND BIOENGINEERING. INCLUDING: SYMPOSIUM BIOTECHNOLOGY IN ENERGY PRODUCTION AND CONSERVATION, JOHN WILEY & SONS. NEW YORK, US, vol. 63, no. 6, 20 June 1999 (1999-06-20), pages 721-729, XP002190336</p> <p>ISSN: 0006-3592</p> <p>page 721 - page 724</p> | 1-29 |
| Y | <p>US 5 788 970 A (VAKHARIA ET AL)</p> <p>4 August 1998 (1998-08-04)</p> <p>cited in the application</p> <p>columns 14-15</p> | 1-29 |
| A | <p>FERNÁNDEZ-ARIAS A ET AL: "Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles"</p> <p>JOURNAL OF GENERAL VIROLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, READING, GB, vol. 79, no. part 5, May 1998 (1998-05), pages 1047-1054, XP002218365</p> <p>ISSN: 0022-1317</p> <p>cited in the application</p> <p>page 1049 - page 1053</p> | 1-29 |
| A | <p>MARTINEZ-TORRECUADRADA J L ET AL:</p> <p>"Different Architectures in the Assembly of Infectious Bursal Disease Virus Capsid Proteins Expressed in Insect Cells"</p> <p>VIROLOGY, ACADEMIC PRESS, ORLANDO, US, vol. 278, no. 2, 20 December 2000 (2000-12-20), pages 322-331, XP004435746</p> <p>ISSN: 0042-6822</p> <p>cited in the application</p> <p>the whole document</p> | 1-29 |
| A | <p>MARTINEZ-TORRECUADRADA J L ET AL:</p> <p>"Structure-dependent efficacy of infectious bursal disease virus (IBDV) recombinant vaccines"</p> <p>VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 21, no. 23, 4 July 2003 (2003-07-04), pages 3342-3350, XP004429746</p> <p>ISSN: 0264-410X</p> <p>the whole document</p> | 1-29 |

| Patent document cited in search report | | Publication date | | Patent family member(s) | Publication date |
|---|---|---------------------|----|----------------------------|---------------------|
| WO 02088339 | A | 07-11-2002 | FR | 2824327 A1 | 08-11-2002 |
| | | | WO | 02088339 A2 | 07-11-2002 |
| <hr/> | | | | | |
| US 5788970 | A | 04-08-1998 | AU | 696656 B2 | 17-09-1998 |
| | | | AU | 2129195 A | 17-10-1995 |
| | | | CA | 2186856 A1 | 05-10-1995 |
| | | | EP | 0755259 A1 | 29-01-1997 |
| | | | JP | 9510873 T | 04-11-1997 |
| | | | US | 6017759 A | 25-01-2000 |
| | | | WO | 9526196 A1 | 05-10-1995 |
| | | | US | 6156314 A | 05-12-2000 |
| <hr/> | | | | | |